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(54) Title: VACCINES FOR ACTINOBACILLUS PLEUROPNEUMONIAE

(57) Abstract

Novel vaccines for use against *Actinobacillus pleuropneumoniae* are disclosed. The vaccines contain at least one *A. pleuropneumoniae* transferrin binding protein and/or one *A. pleuropneumoniae* cytolysin and/or one *A. pleuropneumoniae* APP4. Also disclosed are DNA sequences encoding these proteins, vectors including these sequences and host cells transformed with these vectors. The vaccines can be used to treat or prevent porcine respiratory infections.

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VACCINES FOR ACTINOBACILLUS PLEUROPNEUMONIAE

Technical Field

The instant invention relates generally to the prevention of disease in swine. More particularly, the present invention relates to subunit vaccines for *Actinobacillus pleuropneumoniae*.

Background

Actinobacillus (formerly *Haemophilus*) *pleuropneumoniae* is a highly infectious porcine respiratory tract pathogen that causes porcine pleuropneumonia. Infected animals develop acute fibrinous pneumonia which leads to death or chronic lung lesions and reduced growth rates. Infection is transmitted by contact or aerosol and the morbidity in susceptible groups can approach 100%. Persistence of the pathogen in clinically healthy pigs also poses a constant threat of transmitting disease to previously uninfected herds.

The rapid onset and severity of the disease often causes losses before antibiotic therapy can become effective. Presently available vaccines are generally composed of chemically inactivated bacteria combined with oil adjuvants. However, whole cell bacterins and surface protein extracts often contain immunosuppressive components which make pigs more susceptible to infection. Furthermore, these vaccines may reduce mortality but do not reduce the number of chronic carriers in a herd.

There are at least 12 recognized serotypes of *A. pleuropneumoniae* with the most common in North America

being serotypes 1, 5 and 7. Differences among ser types generally coincide with variations in the electrophoretic mobility of outer membran proteins and enzymes thus indicating a clonal origin of isolates from the same serotype. This antigenic variety has made the development of a successful vaccination strategy difficult. Protection after parenteral immunization with a killed bacterin or cell free extract is generally serotype specific and does not prevent chronic or latent infection. Higgins, R., *et al.*, *Can. Vet. J.* (1985) 26:86-89; MacInnes, J.I. and Rosendal, S., *Infect. Immun.* (1987) 55:1626-1634. Thus, it would be useful to develop vaccines which protect against both death and chronicity and do not have immunosuppressive properties. One method by which this may be accomplished is to develop subunit vaccines composed of specific proteins in pure or semi-pure form.

A. pleuropneumoniae strains produce several cytolysins. *See, e.g.* Rycroft, A.N., *et al.*, *J. Gen. Microbiol.* (1991) 137:561-568 (describing a 120 kDa cytolysin from *A. pleuropneumoniae*); Chang, Y.F., *et al.*, *DNA* (1989) 8:635-647 (describing a cytolysin isolated from *A. pleuropneumoniae* serotype 5); Kamp, E.M., *et al.*, Abstr. *CRWAD* (1990) 1990:270 (describing the presence of 103, 105 and 120 kDa cytolysins in *A. pleuropneumoniae* strains) and Welch, R.A., *Mol. Microbiol.* (1991) 5:521-528 (reviewing cytolysins of gram negative bacteria including cytolysins from *A. pleuropneumoniae*). One of these cytolysins appears to be homologous to the alpha-hemolysin of *E. coli* and another to the leukotoxin of *Pasteurella haemolytica*. Welch, R.A., *Mol. Microbiol.* (1991) 5:521-528. These proteins have a molecular mass of approximately 105 kDa and are protective in mouse and pig animal models against challenge with the homologous serotype. However, cross-serotype protection is limited

at best (Higgins, R., *et al.*, *Can. J. Vet.* (1985) 26:86-89; MacInnes, J.I., *et al.*, *Infect. Immun.* (1987) 55:1626-1634. The genes for two of these proteins have been cloned and expressed in *E. coli* and their nucleotide sequence determined. Chang, Y.F., *et al.*, *J. Bacteriol.* (1991) 173:5151-5158 (describing the nucleotide sequence for an *A. pleuropneumoniae* serotype 5 cytolysin); and Frey, J., *et al.*, *Infect. Immun.* (1991) 59:3026-3032 (describing the nucleotide sequence for an *A. pleuropneumoniae* serotype 1 cytolysin).

Transferrins are serum glycoproteins that function to transport iron from the intestine where it is absorbed, and liver, where it is stored, to other tissues of the body. Cell surface receptors bind ferrotransferrin (transferrin with iron) and the complex enters the cell by endocytosis. *A. pleuropneumoniae*, under iron restricted growth conditions, can use porcine transferrin as its sole iron source, but it cannot utilize bovine or human transferrin (Gonzalez, G.C., *et al.*, *Mol. Microbiol.* (1990) 4:1173-1179; Morton, D.J., and Williams, P., *J. Gen. Microbiol.* (1990) 136:927-933). The ability of other microorganisms to bind and utilize transferrin as a sole iron source as well as the correlation between virulence and the ability to scavenge iron from the host has been shown (Archibald, F.S., and DeVoe, I.W., *FEMS Microbiol. Lett.* (1979) 6:159-162; Archibald, F.S., and DeVoe, I.W., *Infect. Immun.* (1980) 27:322-334; Herrington, D.A., and Sparling, F.P., *Infect. Immun.* (1985) 48:248-251; Weinberg, E.D., *Microbiol. Rev.* (1978) 42:45-66).

It has been found that *A. pleuropneumoniae* possesses several outer membrane proteins which are expressed only under iron limiting growth conditions (Deneer, H.G., and Potter, A.A., *Infect. Immun.* (1989) 57:798-804). Three of these proteins have been isolated

from *A. pleuropneumoniae* serotypes 1, 2 and 7 using affinity chromatography. These proteins have molecular masses of 105, 76 and 56 kDa. (Gonzalez, G.C., *et al.*, *Mol. Microbiol.* (1990) 4:1173-1179). The 105 and 56 kDa proteins have been designated porcine transferrin binding protein 1 (pTfBP1) and porcine transferrin binding protein 2 (pTfBP2), respectively. (Gonzalez, G.C., *et al.*, *Mol. Microbiol.* (1990) 4:1173-1179). At least one of these proteins has been shown to bind porcine transferrin but not transferrin from other species (Gonzalez, G.C., *et al.*, *Mol. Microbiol.* (1990) 4:1173-1179). It is likely that one of these proteins, either alone or in combination with other iron regulated outer membrane proteins, is involved in the transport of iron. The protective capacity of these proteins has not heretofore been demonstrated.

Disclosure of the Invention

The instant invention is based on the discovery of novel subunit antigens from *A. pleuropneumoniae* which show protective capability in pigs.

Accordingly, in one embodiment, the subject invention is directed to a vaccine composition comprising a pharmaceutically acceptable vehicle and a subunit antigen composition. The subunit antigen composition includes at least one amino acid sequence substantially homologous and functionally equivalent to an immunogenic polypeptide of an *Actinobacillus pleuropneumoniae* protein or an immunogenic fragment thereof. The immunogenic protein is selected from the group consisting of *Actinobacillus pleuropneumoniae* transferrin binding protein, *Actinobacillus pleuropneumoniae* cytolysin and *Actinobacillus pleuropneumoniae* APP4.

In other embodiments, the instant invention is directed to a nucleotide sequences encoding

Actinobacillus pl uropneumoniae transferrin binding proteins and nucleotide s quences encoding *Actinobacillus pleuropneumoniae* APP4 proteins, or proteins substantially homologous and functionally equivalent thereto.

In yet other embodiments, the subject invention is directed to DNA constructs comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* transferrin binding protein; and

(b) control sequences that are operably linked to the coding sequence whereby the coding sequence can be transcribed and translated in a host cell, and at least one of the control sequences is heterologous to the coding sequence.

In another embodiment, the subject invention is directed to a DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* cytolysin; and

(b) control sequences that are operably linked to the coding sequence whereby the coding sequence can be transcribed and translated in a host cell, and at least one of the control sequences is heterologous to said coding sequence.

In still another embodiment, the invention is directed to a DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* APP4; and

(b) control sequences that are operably linked to the coding sequence whereby the coding sequence can be transcribed and translated in a host cell, and at least

one of the control sequences is heterologous to the coding sequence.

In still further embodiments, th instant invention is directed to expression cassettes comprising the DNA constructs, host cells transformed with these expression cassettes, and methods of recombinantly producing the subject *Actinobacillus pleuropneumoniae* proteins.

In another embodiment, the subject invention is directed to methods of treating or preventing pneumonia in swine comprising administering to the swine a therapeutically effective amount of a vaccine composition as described above.

In still other embodiments, the invention is directed to isolated and purified *Actinobacillus pleuropneumoniae* serotype 7 60 kDa transferrin binding protein, serotype 5 62 kDa transferrin binding protein, serotype 1 65 kDa transferrin binding protein and serotypes 1 and 5 APP4.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts the nucleotide sequence and deduced amino acid sequence of *A. pleuropneumoniae* serotype 7 60 kDa transferrin binding protein as well as the nucleotide sequence for the flanking regions.

Figure 2 shows the nucleotide sequence and deduced amino acid sequence of *A. pleuropneumoniae* serotype 1 65 kDa transferrin binding protein as well as the nucleotide sequence for the flanking regions.

Figure 3 is a comparison of the amino acid sequences of *A. pleuropneumoniae* serotype 7 60 kDa transferrin binding protein (designated "TF205" therein)

and the *A. pleuropneumoniae* serotype 1 65 kDa transferrin binding protein (designated "TF37" therein). Dots indicate positions of identity.

Figure 4 shows the partial nucleotide sequence of *A. pleuropneumoniae* serotype 7, 103 kDa cytolysin. The *Bgl*II site is the fusion point between the vector pGH432 lacI and the *A. pleuropneumoniae* derived sequence.

Figure 5 shows restriction endonuclease cleavage maps of *A. pleuropneumoniae* serotype 7 cytolysin clones. The *cy*A region contains the structural gene for the cytolysin while *cy*C codes for an activator protein.

Figure 6 shows restriction endonuclease cleavage maps for recombinant plasmids coding for *A. pleuropneumoniae* serotype 1 antigens. 6.1 = rAPP4, 6.2 = pTF37/E1. The heavy line indicates the vector sequence and the coordinates are 0.01 Kb.

Figure 7 shows a physical map and the translational activity of plasmid pTF205/E1 and its deletion derivative, pTF205/E2. (A) The thick line represents DNA of the cloning vehicle (pGH433); tac indicates the location of the tac promoter, and the asterisk indicates stop codons in all three reading frames. The horizontal arrow indicates the location and direction of transcription of the encoded protein; as indicated, this DNA fragment was also used as a probe. (B) Depiction of an SDS gel of the IPTG induced aggregate proteins produced by pTF205/E1 (lane 1) and pTF205/E2 (lane 2); the molecular weight standards (lane 3) are phosphorylase b (97,400), bovine serum albumin (66.20), ovalbumin (45,000), and carbonic anhydrase (31,000).

Figure 8 shows the mean ELISA titers (log) from serum collected from pigs prior to vaccination with fractions from the hot saline extracts from Example 1, at day 24 and day 34 after vaccination. Mean values wer

calculated for each vaccine group. The background level of 2.5-3.0 is normal for *Actinobacillus* free pigs.

Figure 9 shows the mean clinical scores of pigs given fractions from the hot saline extracts described in Example 1. Data for the first three days post challenge are shown. Clinical scores range from 0-4 with 4 indicating death.

Figure 10 depicts the mean body temperature of pigs given fractions from the hot saline extracts described in Example 1. Data for the first three days post challenge are shown. The values presented are degrees centigrade above 39°C.

Figure 11 depicts the mean lung scores of pigs given fractions from the hot saline extracts described in Example 1. Lungs were removed at necropsy and scored for the number and size of Porcine Haemophilus Pleuropneumonia lesions. Results are presented as percent of lung area.

Figure 12 shows the means of clinical response (12A) and body temperature (12B) of pigs challenged with *A. pleuropneumoniae* serotype 7 in trial 1 of Example 6. The numbers on top of the bars represent the number of animals from which the values were obtained.

Figure 13 shows the means of clinical response (13A) and body temperature (13B) of pigs challenged with *A. pleuropneumoniae* serotype 7 in trial 2 of Example 6. The numbers on top of the bars represent the number of animals from which the values were obtained.

Figure 14 shows the nucleotide sequence of the flanking regions of the repeats on λCY76/5. *cyt*A marks the position of the *cyt*A gene, and the sequence at the *Xba*I site and upstream is identical to that described by Chang, Y.F., *et al.*, *DNA* (1989) 8:635-647.

Figure 15 depicts the nucleotide sequence of the inverted repeats of Figure 14 located on either end

of the direct repeats. Complementary bases are connected with a vertical dash.

Figure 16 depicts the nucleotide s quence of the *Bam*HI-*Bgl*II fragment of λCY76Δ1/1. *Bam*HI, *Kpn*I, and *Bgl*II indicate the position of the restriction enzyme sites. The position and direction of the open reading frame is indicated by "MET" and "***". "SD" marks the Shine-Dalgarno consensus sequence. The ends of the repeat are comprised of 26 bp long inverted repeats also emphasized by bold print.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); DNA Cloning, Vols. I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. 1984); Animal Cell Culture (R.K. Freshney ed. 1986); Immobilized Cells and Enzymes (IRL press, 1986); Perbal, B., A Practical Guide to Molecular Cloning (1984); the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

All patents, patent applications and publications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will b employed, and ar intended to be defined as indicated below.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

By "subunit antigen" is meant an antigen entity separate and discrete from a whole bacterium (live or killed). Thus, an antigen contained in a cell free extract would constitute a "subunit antigen" as would a substantially purified antigen.

A "hapten" is a molecule containing one or more epitopes that does not stimulate a host's immune system to make a humoral or cellular response unless linked to a carrier.

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

The terms "immunogenic polypeptide" and "immunogenic amino acid sequence" refer to a polypeptide or amino acid sequence, respectively, which elicits antibodies that neutralize bacterial infectivity, and/or mediate antibody-complement or antibody dependent cell

cytotoxicity to provide protection of an immunized host. An "immunogenic polypeptide" as used herein, includes the full length (or near full length) sequence of th d sired *A. pleuropneumoniae* protein or an immunogenic fragment thereof. By "immunogenic fragment" is meant a fragment of a polypeptide which includes one or more epitopes and thus elicits antibodies that neutralize bacterial infectivity, and/or mediate antibody-complement or antibody dependent cell cytotoxicity to provide protection of an immunized host. Such fragments will usually be at least about 5 amino acids in length, and preferably at least about 10 to 15 amino acids in length. There is no critical upper limit to the length of the fragment, which could comprise nearly the full length of the protein sequence, or even a fusion protein comprising fragments of two or more of the *A. pleuropneumoniae* subunit antigens.

The term "polypeptide" is used in its broadest sense, *i.e.*, any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from a source occurring in nature. Thus, the term "native transferrin binding protein", "native cytolysin" or "native APP4" would include naturally occurring transferrin binding protein, cytolysin or APP4, respectively, and fragments of these proteins. "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (*e.g.*, restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having the sequence homologous to the mRNA).

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences.

A transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3′ terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a ~~transcription initiation site (conveniently defined by~~ mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be

present between a promoter sequence and the coding sequence and the promoter sequenc can still be considered "operably linked" to the coding sequence.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially homologous also refers to sequences showing

identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment und r, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. *See*, *e.g.*, Sambrook *et al.*, *supra*; DNA Cloning, vols I & II, supra; Nucleic Acid Hybridization, *supra*.

The term "functionally equivalent" intends that the amino acid sequence of the subject protein is one that will elicit an immunological response, as defined above, equivalent to the specified *A. pleuropneumoniae* immunogenic polypeptide.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

A composition containing A is "substantially free of" B when at least about 85% by weight of the total of A + B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A + B in the composition, more preferably at least about 95%, or even 99% by weight.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection

(prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy).

B. General Methods

Central to the instant invention is the discovery of certain *A. pleuropneumoniae* proteins able to elicit an immune response in an animal to which they are administered. The antigens, or immunogenic fragments thereof, are provided in subunit vaccine compositions and thus problems inherent in prior vaccine compositions, such as localized and systemic side reactions, as well as the inability to protect against chronic disease, are avoided. The vaccine compositions can be used to treat or prevent *A. pleuropneumoniae* induced respiratory diseases in swine such as porcine pleuropneumonia. The antigens or antibodies thereto can also be used as diagnostic reagents to detect the presence of *A. pleuropneumoniae* infection in a subject. Similarly, the genes encoding the subunit antigens can be cloned and used to design probes for the detection of *A. pleuropneumoniae* in tissue samples as well as for the detection of homologous genes in other bacterial strains. The subunit antigens are conveniently produced by recombinant techniques, as described herein. The proteins of interest are produced in high amounts in transformants, do not require extensive purification or processing, and do not cause lesions at the injection site or other ill effects.

It has now been found that *A. pleuropneumoniae* possesses proteins able to bind transferrin. Specifically, two transferrin binding proteins have been identified in cell free extracts from *A. pleuropneumoniae* serotype 7. These proteins have molecular masses of approximately 60 kDa and 100 kDa, respectively, as determined by SDS PAGE. The 100 kDa protein is seen only

in cells grown under iron restriction and appears to be present in substantial amounts in the outer membrane. The 60 kDa protein is detectable in whole cell lysates and culture supernatants from bacteria grown under iron restricted conditions. This protein is not seen in outer membranes prepared by SDS solubilization. The protein does not appear to be expressed under conditions of heat, ethanol, or oxidative stress. The 60 kDa protein, when separated by nondenaturing PAGE, binds alkaline phosphatase labeled porcine transferrin and exhibits species-specific binding in competitive ELISAs. Congo Red and hemin are able to bind this protein, thereby inhibiting the transferrin binding activity. Southern and Western blot analysis shows that this, or a related protein is also likely present in *A. pleuropneumoniae* serotypes 2, 3, 4, 8, 9, 10 and 11 in addition to serotype 7. A functionally related protein is present in serotypes 1, 5 and 12. The 60 kDa tranferrin binding protein is effective in protecting pigs against *A. pleuropneumoniae* infections. The presence of this protein in culture supernatants and its absence from purified outer membranes indicates that it is different from the iron regulated outer membrane proteins previously described by Deneer and Potter (Deneer, H.G., and Potter, A.A., *Infect. Immun.* (1989) 57:798-804).

The gene encoding the *A. pleuropneumoniae* serotype 7 60 kDa transferrin binding protein has been isolated and the sequence is depicted in Figure 1. The nucleotide sequence including the structural gene and flanking regions consists of approximately 2696 base pairs. The open reading frame codes for a protein having approximately 547 amino acids. The putative amino acid sequence of the 60 kDa protein is also depicted in Figure 1. The recombinantly produced protein is able

to protect pigs from subsequent challenge with *A. pleuropneumoniae*.

The gene encoding an *A. pleuropneumoniae* serotype 5 transferrin binding protein has also been identified and cloned. This gene was cloned by screening an *A. pleuropneunomiae* serotype 5 genomic library with DNA probes from a plasmid which encodes the serotype 7 60 kDa transferrin binding protein (thus suggesting at least partial homology to this protein). When transformed into *E. coli* HB101, the recombinant plasmid expressing the serotype 5 transferrin binding protein gene produced a polypeptide of approximately 62 kDa which reacted with convalescent serum from an *A. pleuropneumoniae* serotype 5-infected pig. The serotype 5 recombinant transferrin binding protein is also able to protect pigs from subsequent challenge with *A. pleuropneumoniae*, as described further below.

A. pleuropneumoniae serotype 1 has also been found to possess a protein which shows 58.3% homology with the serotype 7 60 kDa transferrin binding protein (Figure 3). The nucleotide sequence and deduced amino acid sequence of the serotype 1 transferrin binding protein is shown in Figure 2. The nucleotide sequence including the structural gene and flanking sequences consists of approximately 1903 base pairs. The open reading frame codes for a protein having about 593 amino acids. This protein has a molecular mass of approximately 65 kDa, as determined by SDS PAGE.

As is apparent, the transferrin binding proteins appear to perform the same function (iron scavenging) and exhibit homology between serotypes. Vaccination with one serotype does not always provide cross-protection against another serotype. However, when these transferrin binding proteins are combined with

other subunit antigens, as d scribed below, cross-protection against clinical symptoms becomes possible.

It has also been found that *A. pleuropneumoniae* serotype 7 possesses at least one cytolysin with protective capability. This cytolysin has a molecular mass of approximately 103 kDa, as determined by SDS-PAGE. The gene for this cytolysin has been cloned and a partial nucleotide sequence determined (Figure 4). The partial sequence shows identity with part of the sequence determined for a cytolysin isolated from *A. pleuropneumoniae* serotype 5 (Chang, Y.F., *et al.*, *DNA* (1989) 8:635-647). A carboxy-terminal fragment of this cytolysin, containing 70% of the protein, has been found protective in an experimental pig model.

A. pleuropneumoniae serotypes also possess another protective protein, designated APP4, having a molecular mass of approximately 60 kDa. The genes encoding the proteins from serotypes 1 and 5, respectively, have been cloned. A restriction endonuclease cleavage map for a recombinant plasmid coding for recombinant *A. pleuropneumoniae* serotype 1 APP4 (rAPP4) is shown in Figure 6.1. The gene coding a serotype 5 homolog of APP4 has been cloned from a library screened with DNA probes from the above plasmid. Both the serotype 5 and serotype 1 APP4 proteins afford protection in pigs from a subsequent challenge with *A. pleuropneumoniae*. Other APP4 proteins useful in the present vaccines include immunogenic APP4 polypeptides from additional *A. pleuropneumoniae* serotypes.

The described proteins, or immunogenic fragments thereof, or cell free extracts including the same, can be used either alone or in combination vaccine compositions. Such compositions can contain any combination of the described antigens, such as one or more *A. pleuropneumoniae* transferrin binding proteins

and/or one or more *A. pleuropneumoniae* cytolysins and/or one or more *A. pleuropneumoniae* APP4s. Combination vaccines containing antigens from more than one serotype will provide broad spectrum protection. However, since it has been found that there is little cross-protection against heterologous serotypes when single antigens are used, for best results, serotype 7 antigens should be used for protection against *A. pleuropneumoniae* serotype 7 infections, serotype 1 antigens for protection against serotype 1 infections, serotype 5 antigens for protection against serotype 5 infections, and so on. Furthermore, based on genetic and antigenic differences of the 60 kDa proteins in strains studied, as well as the presence of two different cytolysins in certain serotypes (described further below), vaccines containing more than one of the cytolysins as well as the serotype specific 60 kDa proteins are particularly attractive for providing cross-protection against clinical symptoms.

If synthetic or recombinant proteins are employed, the subunit antigen can be a single polypeptide encoding several epitopes from just one of the *A. pleuropneumoniae* proteins or several epitopes from more than one of the proteins (*e.g.*, a fusion protein). Synthetic and recombinant subunit antigens can also comprise two or more discrete polypeptides encoding different epitopes.

The above described antigens can be produced by a variety of methods. Specifically, the antigens can be isolated directly from *A. pleuropneumoniae*, as described below. Alternatively, the antigens can be recombinantly produced as described herein. The proteins can also be synthesized, based on the described amino acid sequences, using techniques well known in the art.

For example, the antigens can be isolated from bacteria which express the same. This is generally

accomplished by first preparing a crude extract which lacks cellular components and several extraneous proteins. The desired antigens can then be further purified i.e. by column chromatography, HPLC, immunoadsorbent techniques or other conventional methods well known in the art.

Purification of the above proteins as described herein permits the sequencing of the same by any of the various methods known to those skilled in the art. For example, the amino acid sequences of the subject proteins can be determined from the purified proteins by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art. Furthermore, fragments of the proteins can be tested for biological activity and active fragments, as described above, used in compositions in lieu of the entire protein.

Once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen DNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. *See, e.g.*, DNA Cloning: Vol. I, supra; Nucleic Acid Hybridization, *supra*; Oligonucleotide Synthesis, *supra*; T. Maniatis *et al.*, *supra*.

First, a DNA library is prepared. The library can consist of genomic DNA from *A. pleuropneumoniae*. Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the gene encoding the desired protein. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as

to correspond to the codons encoding a known amino acid sequence from the desired protein. Since the genetic code is d generate, it will often be necessary to synthesize several olig nucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein of interest. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment. Automated oligonucleotide synthesis has made the preparation of large families of probes relatively straightforward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an

evolutionarily close r distant species. The selection of the appropriate conditions is within the skill of the art. *See, generally,* Nucleic Acid hybridization, *supra*. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; *i.e.*, hybridization is due to a sufficient degree of nucleic acid homology (*e.g.*, at least about 65%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular insert contains a gene coding for the desired protein.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. *See, e.g.*, Edge (1981) *Nature* 292:756; Nambair *et al.* (1984) *Science* 223:1299; Jay *et al.* (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria),

pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). *See, generally,* DNA Cloning: Vols. I & II, *supra*; T. Maniatis *et al.*, *supra*; B. Perbal, *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The subunit antigens of the present invention can be expressed using, for example, the *E. coli* tac promoter or the protein A gene (*spa*) promoter and signal sequence. Signal sequences can be removed by the bacterial host in post-translational processing. *See, e.g.*, U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate r gulatory sequences, the positioning and orientation of the coding sequence with respect to the

control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (*i.e.*, RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; *i.e.*, to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the antigens of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. *See, e.g.*, T. Maniatis *et al.*, *supra*; DNA Cloning, Vols. I and II, *supra*; Nucleic Acid Hybridization, *supra*.

A number of procaryotic expression vectors are known in the art. *See, e.g.*, U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; *see also* U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395.

Yeast expression vectors are also known in the art. *See, e.g.*, U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; *see also* European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform *E. coli* and pooling and screening individual colonies using polyclonal serum or monoclonal antibodies to the desired antigen.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The proteins of the present invention or their fragments can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (*e.g.*, mouse, rabbit, goat,

horse, etc.) is immunized with an antigen of the present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. *See, e.g.*, M. Schreier *et al.*, Hybridoma Techniques (1980); Hammerling *et al.*, Monoclonal Antibodies and T-cell Hybridomas (1981); Kennett *et al.*, Monoclonal Antibodies (1980); *see also* U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the antigen of interest, or fragment thereof, can be screened for various properties; *i.e.*, for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against.

Animals can be immunized with the compositions of the present invention by administration of the protein of interest, or a fragment thereof, or an analog thereof. If the fragment or analog of the protein is used, it will include the amino acid sequence of an epitope which interacts with the immune system to immunize the animal to that and structurally similar epitopes. If

combinations of the described antigens are used, the antigens can be administered together or provided as s parate entities.

Prior to immunization, it may be desirable to increase the immunogenicity of the particular protein, or an analog of the protein, or particularly fragments of the protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the antigenic peptide may be administered linked to a carrier. For example, a fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651, and incorporated

herein by reference. Also useful is a fusion product of a viral protein and the subject immunogens made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

The novel proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel proteins can be constructed as follows. The DNA encoding the particular protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the viral genome. The resulting TK^- recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

It is also possible to immunize a subject with a protein of the present invention, or a protective fragment thereof, or an analog thereof, which is administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are

prepared as inj ctables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also b emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. *See, e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the individual being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example,

pharmaceutical grades of mannitol, lactose, starch, magnesium, stearat , sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The proteins can also be delivered using implanted mini-pumps, well known in the art.

Furthermore, the proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and

the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, the polypeptide of interest, or an immunologically active fragment thereof, is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal delivery, are also acceptable. Injectable vaccine formulations will contain an effective amount of the active ingredient in a vehicle, the exact amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. With the present vaccine formulations, 5 μg to 1 mg of active ingredient, more preferably 10 μg to 500 μg, of active ingredient, should be adequate to raise an immunological response when a dose of 1 to 2 ml of vaccine per animal is administered. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the particular antigen or fragment thereof, or analog thereof, in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity to pneumonia.

An alternative route of administration involves gene therapy or nucleic acid immunization. Thus, nucleotide sequences (and accompanying regulatory elements) encoding the subject proteins can be administered directly to a subject for *in vivo* translation thereof. Alternatively, gene transfer can be accomplished by transfecting the subject's cells or tissues *ex vivo* and reintroducing the transformed material into the host. DNA can be directly introduced into the host organism, i.e. by injection (see International Publication No. WO/90/11092; and Wolff et al., Science (1990) 247:1465-1468). Liposome-mediated gene transfer can also be accomplished using known methods. See, e.g., Hazinski et al., Am. J. Respir. Cell Mol. Biol. (1991) 4:206-209; Brigham et al., Am. J. Med. Sci. (1989) 298:278-281; Canonico et al., Clin. Res. (1991) 39:219A; and Nabel et al., Science (1990) 249:1285-1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and cells susceptible to *A. pleuropneumoniae* infection.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were

paid. Access to said cultures will be available during pendency of the patent application to on det rmined by the Commissioner to be ntitled th reto und r 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

These deposits are provided merely as a convenience to those of skill in the art, and are not an admission that a deposit is required under 35 USC §112. The nucleic acid sequences of these plasmids, as well as the amino sequences of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description herein. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

Strain	Deposit Date	ATCC No.
pTF37/E1 (in *E. coli*)	10/19/91	68823
pTF205/E1 (in *E. coli*)	10/19/91	68821
pTF205/E2 (in *E. coli*)	10/19/91	68822
pTF213/E6 (in *E. coli*)	10/8/92	69084
pCY76/503 (in *E. coli*)	10/19/91	68820
p#4-213-84 (in *E. coli*)	10/8/92	69082
prAPP4 (in *E. coli*)	4/7/92	68955
A. pleuropneumoniae serotype 7 strain AP37	10/19/91	55242

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T_4 DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double stranded DNA fragments were separated on agarose gels.

Bacterial Strains, Plasmids and Media

A. pleuropneumoniae serotype 7 strain AP205 was a Nebraska clinical isolate obtained from M.L. Chepok, Modern Veterinary Products, Omaha, Nebraska. *A. pleuropneumoniae* serotype 1 strain AP37, *A. pleuropneumoniae* serotype 5 strain AP213 and *A.*

pleuropneumoniae serotype 7 strain AP76, were isolated from the lungs of diseased pigs giv n to the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The other *A. pleuropneumoniae* strains were field isolates from herds in Saskatchewan. The *E. coli* strain HB101 (*hsd*M, *hsd*R, *rec*A) was used in all transformations using plasmid DNA. *E. coli* strains NM538 (*sup*F, *hsd*R) and NM539 (*sup*F, *hsd*R, P2cox) served as hosts for the bacteriophage λ library. The plasmids pGH432 and pGH433 are expression vectors containing a *tac* promoter, a translational start site with restriction enzyme sites allowing ligation in all three reading frames followed by stop codons in all reading frames.

A. pleuropneumoniae strains were grown on PPLO medium (Difco Laboratories, Detroit, MI) supplemented with 1% IsoVitalex (BBL Microbiology Systems, Becton Dickinson & Co., Cockeysville, MD 21030). Plate cultures were incubated in a CO_2-enriched (5%) atmosphere at 37°C. Liquid cultures were grown with continuous shaking at 37°C without CO_2 enrichment.

Iron restriction was obtained by adding 2,2 dipyridyl to a final concentration of 100 μmol. Heat stress was induced by transferring cultures to 45°C for 2 hours. Ethanol stress was exerted by the addition of 10% (vol/vol final concentration) of absolute ethanol to cultures in mid log phase. Oxidative stress was induced by the addition of 1% (vol/vol final concentration) of 30% H_2O_2 to the cultures. *E. coli* transformants were grown in Luria medium (Maniatis, T., *et al.*, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) supplemented with ampicillin (100 mg/l).

Preparation and Analysis of Culture Supernatants, Outer Membranes and Protein Aggregates.

Cultur supernatants were mixed with two volumes of absolute ethanol and kept at -20°C for 1 h. Precipitates were recovered by centrifugation and resuspended in water. Outer membranes were prepared by sarkosyl solubilization as previously described (Deneer, H.G., and Potter, A.A., *Infect. Immun.* (1989) 57:798-804). For the preparation of protein aggregates, broth cultures (50 ml) in mid log phase (OD_{660} of 0.6) were induced by the addition of 1 mmol isopropylthiogalactoside (IPTG; final concentration). After 2 hours of vigorous shaking at 37°C, cells were harvested by centrifugation, resuspended in 2 ml of 25% sucrose, 50 mmol Tris/HCl buffer pH 8, and frozen at -70°C. Lysis was achieved by the addition of 5 μg of lysozyme in 250 mmol Tris/HCl buffer pH 8 (5 min on ice), addition of 10 ml detergent mix (5 parts 20 mmol Tris/HCl buffer pH 8 (5 min on ice), addition of 10 ml detergent mix (5 parts 20 mmol Tris/HCl buffer pH 7.4, 300 mmol NaCl, 2% deoxycholic acid, 2% NP-40, and 4 parts of 100 mmol Tris/HCl buffer pH 8, 50 mmol ethylenediamine tetraacetic acid, 2% Triton X-100), and by sonication. Protein aggregates were harvested by centrifugation for 30 min at 15,000 g. Aggregate protein was resuspended in H_2O to a concentration of 5-10 mg/ml and solubilized by the addition of an equal volume of 7 molar guanidine hydrochloride.

Proteins were analyzed by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) according to the method of Laemmli (Laemmli, M.K., *Nature* (1970) 227:680-685). The protein concentration was determined using a modified Lowry protein assay which prevents reaggregation of the protein. Bovine serum albumin (Pierce Chemical Co., Rockford, IL) was used as a

standard. Briefly, samples were taken up in 0.5 ml of 1% sodium dodecyl sulfate (SDS), 0.1 mol NaOH, and 1.5 ml of 0.2 mol Na_2CO_3, 0.07 mol $NaKC_4H_4O_6 \cdot 4H_2O$, 0.1 mol NaOH, 0.001 mol $CuSO_4 \cdot 5H_2O$ wer added. After 15 min incubation at 20°C, 0.15 ml of phenol reagent, diluted 1:2 with distilled water, was added. Samples were incubated at 55°C for 15 min, and the optical density at 660 nm was determined.

Electrophoretic transfer onto nitrocellulose membranes was performed essentially as described by Towbin *et al.* (Towbin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1979) 76:4350-4354). Nonspecific binding was blocked by incubation in 0.5% gelatine in washing buffer (150 mmol saline, 30 mmol Tris-HCl, 0.05% Triton-X100). Antibody and alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added in washing buffer, and each incubated for 1 h at room temperature. Blots were developed with a substrate containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT); ImmunoSelect, BRL, Gaithersburg, MD) in 100 mmol Tris/HCl buffer pH 9.5, 50 mmol NaCl, 5 mmol $MgCl_2$.

Preparation of Antisera

Convalescent serum was obtained as follows. Pigs were given 10^3 *A. pleuropneumoniae* intranasally and were challenged 2 weeks later with 2 LD50. Serum against the recombinant protein was raised in mice by intraperitoneal injection of 30 μg of solubilized aggregate in complete Freund's adjuvant and a subcutaneous boost with 30 μg protein in incomplete Freund's adjuvant two weeks later.

Iron Compounds

Transferrins from different species were obtained commercially (porcine transferrin from The Binding Site, Birmingham, UK; human and bovine transferrin from Sigma Chemical Co.). Porcine transferrin was iron depleted as described by Mazurier and Spik (Mazurier, J., and G. Spik, *Biochim. Biophys. Acta* (1980) 629:399-408). The resulting porcine apotransferrin as well as the commercially obtained bovine and human apotransferrins were iron repleted as described by Herrington and Sparling (Herrington, D.A., and F.P. Sparling, *Infect. Immun.* (1985) 48:248-251).

Transferrin Binding Assays

To assess the possible transferrin binding ability of recombinant proteins, a Western blot-like transferrin binding assay was performed essentially as described by Morton and Williams (Morton, D.J., and P. Williams, *J. Gen. Microbiol.* (1990) 136:927-933). During the entire procedure the temperature was kept below 37°C. Blots were developed using biotinylated transferrin (Biotin-XX-NHS Ester Labeling Kit, Clontech Laboratories, Palo Alto, CA) coupled to streptavidin phosphatase and purified by gel filtration using a G-100 column. In order to determine species specificity of transferrin binding, a competitive ELISA was developed. ELISA plates (Immulon 2, Dynatech Laboratories, McLean, Virginia) were coated with 100 μl of porcine transferrin at a concentration of 100 μg/ml in carbonate buffer at 4°C over night. All subsequent steps were performed at room temperature. Plates were blocked with 0.5% gelatine in washing buffer. Solubilized protein at a concentration of approximately 5 μg/ml was incubated in washing buffer for 1 hour with an qual volume of serial two fold dilutions of porcine, bovine, and human transferrin.

Subsequently, 200 μl of this solution were added to the coated and washed wells and incubated for one hour. The assay was developed using a mouse serum raised against the recombinant protein, an alkaline phosphatase labeled conjugate and p-nitrophenyl phosphate in 1 mol diethanolamine, pH 9.5, 5 mmol $MgCl_2$ as substrate. The plates were read at 405 nm in a Biorad plate reader, and 50% inhibition values were determined for the various transferrins.

EXAMPLES

Example 1
Fractionation of Hot Saline Extracts

Vaccination of pigs with cell free extracts reduces mortality following experimental challenge. However, the presence of an uncharacterized immunosuppressive component can interfere with the induction of protective immunity in a dose dependent fashion. Therefore, an attempt was made to remove this component by preparative isoelectrofocusing. Cell free extracts were prepared as follows. *Actinobacillus pleuropneumoniae* serotype 1 strain AP37 was grown to mid log phase in PPLO broth supplemented with Isovitalex and the bacteria harvested by pelleting cells by centrifugation at 8,000 x g for 15 minutes. Cells were resuspended in 1/10 volume of 0.85% sodium chloride and the mixture was shaken with glass beads at 60°C for 1 hour. Cells were removed by centrifugation as described above and the supernatant material filter sterilized. This material was dialyzed against distilled water to remove the sodium chloride, mixed with Biorad ampholytes (pH range 3-11) and loaded in a Rotafor isoelectrofocusing cell. The mixture was focused at 12 watts constant power for 4-6 hours. Fractions were pooled into four samples according

to pH as shown below. This material was used to vaccinate groups of 6 pigs as shown below.

<u>Group 1</u>: Fraction A, pH = 10.4
<u>Group 2</u>: Fraction B, pH = 6.1
<u>Group 3</u>: Fraction C, pH = 5.2
<u>Group 4</u>: Fraction D, pH = 2.4
<u>Group 5</u>: Mixture, Fraction A-D
<u>Group 6</u>: Same as Group 5.
<u>Group 7</u>: Placebo (no antigen)

Marcol-52 was used as an adjuvant, and all pigs were boosted with the appropriate vaccine formulation after 3 weeks. After an additional week, all pigs were exposed to an aerosol of *Actinobacillus pleuropneumoniae* strain AP37 and clinical data plus body temperatures were recorded daily. In addition, serum samples collected at days 0, 21 and 34 of the trial were used to determine the serological response to vaccination by an enzyme linked immunosorbent assay (ELISA). The results are summarized in Figures 8 through 11. Pigs in Groups 1, 4, 5 and 6 all had significantly increased ELISA titers compared to the control group while those in Group 2 and 3 were only marginally better. These results were reflected in the mean clinical scores (Figure 9), mean temperatures (Figure 10) and mean lung scores (Figure 11). Clearly, those pigs which received Fraction D or the mixture of all four Fractions were protected against experimental challenge. Furthermore, it appeared that these vaccine preparations reduced colonization of the lung, which can be a measure of chronicity.

Each of the above fractions was analyzed by polyacrylamide gel electrophoresis and Western blotting using sera collected from each pig prior to challenge. Fractions A and B contained little protein but a

substantial quantity of lipopolysaccharide and lipoprotein. Fraction C contained a small quantity of protein, largely four components with molecular weights ranging from 100,000 to 14,000. Fraction D, which exhibited the greatest protective capacity, had the largest quantity of protein and contained at least 22 different components. However, only 7 proteins were present in significant amounts. Western blots revealed the presence of four strongly reactive proteins in Fractions C and D. These proteins had molecular weights of approximately 20 kDa, 40 kDa, 75 kDa and 100 kDa.

Example 2

Cloning of Genes Coding for Serotype 1 Protective Proteins

All restriction enzyme digests were done in T4 DNA polymerase buffer (Maniatis, T., *et al.*, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) containing 1 mmol dithiothreitol and 3 mmol spermidine.
A. pleuropneumoniae AP37 genomic DNA was prepared as previously described (Stauffer, G.V., *et al.*, *Gene* (1981) 14:63-72) and partially digested with the restriction endonuclease *Sau*3AI. Fragments of 3000 to 8000 Bp were isolated by sucrose density gradient centrifugation (Maniatis, supra) and ligated into pGH432 and pGH433 which had been digested with *Bam*HI and/or *Bgl*II. The ligated DNA was used to transform *E. coli* strain JM105. The colonies were transferred to nitrocellulose membranes, induced with IPTG and screened for reaction with serum from pigs vaccinated with Fraction D of the hot saline extract (above). Three positive clones which expressed Actinobacillus proteins were selected for further study. The restriction endonuclease maps of the three plasmids are shown in Figure 6. One clone, prAPP4

(Figure 6.1), codes for the serotype 1 APP4. Another clone (pTF37/E1, Figure 6.3) codes for a putative serotype 1 transferrin binding pr tein, based n homology with its serotype 7 homolog (see below and Figure 3). The nucleotide sequence of the gene coding for this protein was determined using the chain termination method as described by Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA* (1977) 74:5463-5467. Nested deletions were prepared by exonuclease III treatment, and specific primers were prepared using a Pharmacia Gene Assembler. Sequences were analyzed using the IBI/Pustell program and the Genbank network. The nucleotide sequence and deduced amino acid sequence are depicted in Figure 2.

Example 3

Cloning of *Actinobacillus pleuropneumoniae* Serotype 7 60 kDa Transferrin Binding Protein

As above, all restriction enzyme digests were done in T4 DNA polymerase buffer (Maniatis, *supra*) containing 1 mmol dithiothreitol and 3 mmol spermidine. Genomic DNA libraries of *A. pleuropneumoniae* serotype 7 strain AP205 were prepared as previously described (Stauffer, *supra*) and partially digested with the restriction endonuclease *Sau*3AI. Fragments of 1500 to 2500 Bp were isolated by sucrose density gradient centrifugation (Maniatis, *supra*) and ligated into pGH432 and pGH433. *E. coli* HB101 transformants were replica plated onto nitrocellulose membranes, induced for 2 hours on plates containing 1 mM IPTG and screened for reaction with serum from pigs infected with serotype 7 *A. pleuropneumoniae*. Positive transformants were replated, induced with IPTG and whole cell proteins were analyzed by Western blotting. A whole cell lysate of *A. pleuropneumoniae* grown under iron limiting conditions was used as a control.

Of approximately 6000 transformants screened by immunoblotting, 22 reacted with convalescent serum and showed an immun reactive band in the Western blot analysis. One transformant expressed a protein with the same electrophoretic mobility as an *A. pleuropneumoniae* polypeptide present only under iron limiting growth conditions. The plasmid present in this transformant was designated pTF205/E1 (Figure 7A). The recombinant polypeptide produced by this strain had a molecular weight of 60,000 (Figure 7B) and was produced as inclusion bodies, indicating that it was under the control of the *tac* promoter. Aggregated protein prepared from pTF205/E2 (a BamHI/BglII deletion derivative of the original plasmid) was used to immunize mice. The resulting serum reacted with a single polypeptide in the whole cell lysates and in culture supernatants from *A. pleuropneumoniae* serotype 7 strain AP205 grown under iron limiting conditions. Outer membranes prepared by sarkosyl solubilization (Deneer, H.G., and Potter, A.A., *Infect. Immun.* (1989) 57:798-804) of cells grown under iron limiting conditions did not react with the antiserum. Likewise, whole cell lysates, culture supernatants and outer membranes from cells grown in iron replete media did not react with the antibody.

The recombinant protein separated by non-reducing polyacrylamide gel electrophoresis was found to bind alkaline phosphatase-labeled porcine transferrin. This binding was shown to be species specific in a competitive ELISA, where the binding of the solubilized protein to iron replete porcine transferrin could be inhibited completely only by iron replete porcine transferrin. Porcine apotransferrin also inhibited binding, but a higher concentration was necessary. Using human and bovine iron-deplete and -replete transferrins, 50% inhibition could not be obtained even with

concentrations 40 times higher than the inhibitory dose for porcine transferrin. In addition, relatively high concentrations of both hemin and Congo Red could inhibit transferrin-binding of the 60 kDa protein, whereas porcine hemoglobin, EDDA, dipyridyl, and ferric citrate failed to do so (Table 1).

Congo Red and hemin binding by *E. coli* transformants expressing this protein at low levels was detected by supplementing the ampicillin containing Luria agar with 1-10 μmol IPTG and 0.003% Congo Red or 0.02% hemin.

Table 1

Competitive ELISA Showing the Differences in Affinity of the Recombinant 60 kDa Protein Toward Transferrins of Various Species

Solid Phase Antigen	Competitive Substances[1]	50% Inhibition Values[2] [µg/ml]	[µmol]
porcine transferrin (TF)	porcine	25[3]	0.3
	porcine aTF	150	1.8
	human TF/aTF	>1000[4]	>12.5
	bovine TF/aTF	>1000[4]	>12.5
	porcine TF, NH_2-terminus	20	0.5
	bovine hemin	4	6.0
	Congo Red	25	35.0

[1] Also tested and completely noninhibitory were porcine hemoglobin (14 µmol), EDDA (100 µmol, iron-saturated), Dipyridyl (100 µmol, iron-saturated), and ferric citrate (10 mmol).

[2] Inhibition values state the concentration of transferrin necessary in the preincubation step in order to obtain an inhibition of 50% in the reaction between recombinant protein and solid phase transferrin.

[3] The value varied between different experiments between 12.5 and 100 µg/ml; however, the relative difference in inhibitory activity between the various substances was constant.

[4] This concentration had an inhibitory effect, but it was below 50%.

Chromosomal DNA was prepared from 27 different clinical isolates of *A. pleuropneumoniae* belonging to 6 different serotypes digested with the restriction endonucleases *Bgl*II and *Eco*RV, and separated on an agarose gel. This fragment was chosen because the functional activity of the deletion plasmid pTF205/E2 localized the position of the serotype 7 60 kDa gene upstream of the *Bgl*II site. A Southern blot analysis using the *Eco*RV/*Bgl*II fragment of pTF205/E1 as a probe detected a fragment identical in size in all of the above *A. pleuropneumoniae* serotype 2, 4 and 7 strains as well as in one serotype 3 strain. In contrast, none of the serotype 1 and 5 strains reacted with the probe. Neither did the *E. coli* HB101 and *Pasteurella haemolytica* controls.

The nucleotide sequence of the gene coding for the transferrin binding protein was determined by the chain termination method as described in Example 2 and is shown in Figure 1.

Example 4

Cloning of *A. pleuropneumonia* Serotype 7 Cytolysin Gene

A recombinant plasmid containing the carboxy-terminal 70% of the 103 kDa serotype 7 cytolysin gene (*cyt*A) was constructed as follows. A gene library of *A. pleuropneumoniae* serotype 7 strain AP76 was constructed in the phage vector λ2001. Plaques were screened by hybridization using the *Pasteurella haemolytica lkt*A gene as a probe (see Lo, R.Y.C., *et al.*, *Infect. Immun.* (1987) 55:1987-1996 for a description of this gene). Positive plaques were purified and a 16 kb *Eco*RI fragment was subcloned into the plasmid vector pACYC184 (plasmid pCY76/5, Figure 5). A 3.5 kb *Bgl*II fragment from pCY76/5 was further subcloned into the *Bgl*II site of the expression vector pGH432 *lacI* which

provides a 5 amino acid leader peptide and an IPTG inducible promoter (pCY76/503, Figure 5). Nucleotide sequence analysis of th fusion site reveal d s quence identity with the cytolysin from *A. pleuropneumoniae* serotype 5 (Figure 4; Chang, Y.F., *et al.*, *DNA* (1989) 8:635-647). Further analysis of the *A. pleuropneumoniae* cytolysin type II genes by Southern blotting revealed that the B and D genes are not located immediately downstream from the *cytA* gene on the Actinobacillus chromosome. This is unusual, as the cytolysin C, A, B and D genes are clustered in the *A. pleuropneumoniae* cytolysin type I (Frey, J., and Nicolet, J., *J. Clin. Microbiol.* (1990) 28:232-236), *P. haemolytica* leukotoxin (Strathdee, C.A. and Lo, R.Y.C., *Infect. Immun.* (1989) 171:916-928), and the *E. coli* alpha hemolysin (Welch, R.A. and Pellet, S.A. *J. Bacteriol.* (1988) 170:1622-1630).

E. coli HB101 containing plasmid pCY76/503 expressed the recombinant cytolysin (CytA) as inclusion bodies upon induction with IPTG. The protein made up 30% of the total protein content in the pCY76/503 transformants. Isolated protein aggregates were estimated to be 75% pure. The resulting protein could be detected by *A. pleuropneumoniae* convalescent serum and by antibodies raised against the *A. pleuropneumoniae* type 1 cytolysin-containing culture supernatant. Restriction endonuclease maps of the cytolysin gene and sequence data are shown in Figures 5 and 4.

Example 5

Isolation and Characterization of Spontaneous Mutants of th cytA Gene

Spontaneous deletions of the *cyt*A gene from the *A. pleuropneumoniae* chromosome occur at high frequency (approximately 1/10,000 colonies), as determined by reaction with monospecific antisera against the cytolysin. In order to isolate and characterize the spontaneous mutants, *A. pleuropneumoniae* strains AP76 and AP205 were subcultured twice from single colonies. Two independent serial dilutions were made for each strain, and from each approximately 10,000 colonies were plated. After replica-plating onto nitrocellulose, three independent cytolysin-negative colonies were detected by immunoblot and designated AP76Δ1, AP205Δ1, and AP205Δ2. Western blot analysis of whole cell lysates revealed that these colonies lacked the cytolysin whereas the Coomassie blue stained total protein profile appeared to be identical with the wildtype. Southern blot analysis of restricted DNA from AP76Δ1 and AP205Δ1 with λCY76/5-derived probes revealed that the *Bgl*II fragment was absent, although hybridization was observed after using the *Bgl*II fragment as a probe. Hybridization with the *Bgl*II-*Eco*RI fragments located on either end of λCY76/5 resulted in the appearance of strong bands in the cytolysin-negative mutants, and the hybridizing *Eco*RI fragment appeared to be approximately 7 kb smaller than that in the wildtype.

In order to characterize the *cyt*A excision site, a genomic library was prepared from AP76Δ1 and probed with the *Eco*RI fragment derived from λCY76/5. Several clones were isolated, and initial characterization revealed that one clone had a *Bam*HI-*Kpn*I fragment identical in size to that of λCY76/5. This clone was designated as λCY76Δ1/1. Also, the nucleotide

sequence of the *BamHI-KpnI* fragment of this clone was identical to the corresponding region of λCY76/5. Part of this sequence was present a second time on λCY76/5 starting 358 bp downstream fr m the end of *cytA* (Figures 14 and 15). Further analysis showed that *cytA* is flanked by two identical direct repeats each being 1201 bp in length, and that one repeat is completely conserved in λCY76/Δ1. The sequence flanking the direct repeats located on either site of the *cytA* gene in λCY76/5 is TTAATG---AATATT, and this sequence does not comprise part of an apparent longer reading frame (Figure 16). An initial analysis of the repeat sequence revealed that its ends form complementary repeats with 4 mismatches over a length of 26 bp. They also contain one open reading frame going in the opposite direction than *cytA*. The open reading frame is 1038 nucleotides long and preceded by a Shine-Dalgarno consensus sequence.

Example 6

The Protective Capacity of Serotype 7 Recombinant Proteins

E. coli HB101 strains expressing the transferrin binding protein and the 103 kDa cytolysin were grown to mid log phase in 50 ml broth cultures and induced by the addition of 2 mM IPTG. After two hours of vigorous shaking at 37°C, cells were harvested by centrifugation and resuspended in 2 ml 50 mM of Tris-HCl, pH 8, 25% sucrose, and frozen at -70°C. The cell suspension was thawed, 5 μg of lysozyme added and after 5 min on ice, 10 ml of detergent mix was added to lyse cells. The lysed cell suspension was sonicated to reduce viscosity and protein aggregates were harvested by centrifugation for 30 min at 15,000 g. The aggregated protein was resuspended in double distilled water to a concentration of 5-10 mg/ml and solubilized by the

addition of an equal volume of 7 M guanidine hydrochloride. The solubilized protein was diluted in distilled water to 1 mg/ml and emulsified in Amphigen (Smith-Kline Beecham, Lincoln, NE) with Tween80 (Sigma Chemical Co., St. Louis, MO) and Span (Sigma Chemical Co., St. Louis, MO) using a Polytron homogenizer (Kinematica GmbH, Littau, Switzerland). Each 2 ml dose of vaccine contained Amphigen (100 μl), Tween80 (28 μl), Span (12 μl), guanidine hydrochloride (20 mmol), and protein as indicated below.

Trial 1: 48 pigs were randomly assigned to 8 groups and immunized by intramuscular injection in the neck muscle twice (on days 1 and 21) as follows: 2 groups received 25 μg of recombinant CytA, 2 groups received 25 μg of recombinant *A. pleuropneumoniae* serotype 7 60 kDa protein, 2 groups received both proteins, and 2 groups (unimmunized controls) received the adjuvant only. One set of 4 groups was subsequently challenged on day 32 with *A. pleuropneumoniae* serotype 1 strain AP37 (4.1×10^5 CFU/ml), the other one with *A. pleuropneumoniae* serotype 7 strain AP205 (1.4×10^8 CFU/ml).

Trial 2: 24 pigs were randomly assigned to 4 groups, and the groups twice received 0, 12.5, 50, or 200 μg recombinant *A. pleuropneumoniae* serotype 7 60 kDa protein. Subsequently, all groups were challenged with 7×10^8 CFU/ml of *A. pleuropneumoniae* serotype 7 strain AP205.

Clinical data plus body temperatures were recorded daily for 3 days post challenge and each animal received a daily average clinical score. The scoring system is defined as follows: 0 - clinically normal; 1 - slight increase in respiratory rate and effort, slight depression; 2 - marked increase in respiratory rate and effort, marked depression; 3 - severe increase

in respiratory rate and effort, severe depression, mouth breathing and/or cyanotic. Animals with a clinical score f 3 were euthanized.

In addition, serum samples collected at days 0, 21 and 28 of the trial were used to determine the serological response to vaccination by an enzyme linked immunosorbent assay (ELISA). All serum samples were titrated in the ELISA against the recombinant serotype 7 60 kDa transferrin binding protein, the recombinant cytolysin protein, as well as against an *A. pleuropneumoniae* serotype 7 and serotype 1 extract (Willson, P.J., *et al.*, *Can. Vet. J.* (1988) 29:583-585). Briefly, plates were coated overnight at 4°C with 100 μl of a solution containing either 1 μg/ml of recombinant protein or 10 μg/ml of extract protein in carbonate buffer. Plates were blocked for 1 h at room temperature with 0.5% gelatine in washing buffer (150 mmol saline, 30 mmol Tris-HCl, 0.05% Tween20). An internal standard consisted of a pool of equal volumes of swine antisera to *A. pleuropneumoniae* serotype 1 and serotype 7 that was diluted 1:100 in washing buffer. Serum dilutions and goat-anti-pig alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were each left to incubate for 1 h at room temperature. Plates were developed at 37°C with 100 μl p-nitrophenyl phosphate (3 g/l) in 1 mol diethanolamine, 50 mmol $MgCl_2$, pH 9.8. The development time was varied for the different coating antigens such that the control serum had a titer between 1:800 and 1:1600 (10 min for the cytolysin, 20 min for the *A. pleuropneumoniae* serotype 1 extract, 45 min for the 60 kDa protein, 90 min for the *A. pleuropneumoniae* 7 extract).

The trials were terminated on day 40, and all surviving pigs were euthanized. The injection sites were examined, and lungs were scored to determine the

percentage of pulmonary ar a affected by lesions using a computerized digitizer. Lungs were cultured to determine the presence of *A. pleuropneumoniae* and to confirm its serotype.

The significance of the difference in mortality rates among the different groups was determined using a G^2 likelihood ratio test (Dixon, W.J., *et al.*, BMDP Statistical Software Manual, University of California Press, 1988, pp. 229-273.

The results are summarized in Tables 2 and 3. As can be seen, all pigs in Trial 1 developed a strong antibody response to the recombinant antigen with which they had been immunized (Table 2). There was a significant difference ($p < 0.03$) in mortality among the 8 groups. After challenge with *A. pleuropneumoniae* serotype 7 (strain AP205), the mortality in all immunized groups was lower than in the control group ($p < 0.1$). Also, the damage to the lungs of immunized pigs may be less extensive than that seen in the control pigs (Table 2). This outcome was reflected by a generally milder course of disease shown by lower body temperature and clinical scores during the first 3 days after challenge (Figures 12A and 12B). Pigs that developed an antibody response against both recombinant antigens showed a particularly mild course of disease (Figures 12A and 12B), and damage to their lungs was minimal (Table 2).

All pigs in trial 2 developed a strong antibody response to the 60 kDa protein, and the titers were independent of the dose (Table 3). The immunized groups had a lower mortality than the control group ($p = 0.14$), and the lesion score of the lungs from pigs in group H was also reduced for immunized pigs (Table 2). These results are supported by the clinical data obtained in the first 3 days after challenge (Figures 13A and 13B).

Both mortality and clinical data do not show an increased efficacy of th higher antigen dose.

In both trials, the injection sites were free of macroscopically detectable alterations. In all pigs, *A. pleuropneumoniae* was isolated from the lungs 1 week after challenge.

In agreement with previous findings, our results show a lack of protection against a heterologous serotype despite an appreciable serum titer in the animals (Table 2). This lack of cross-protection could be explained by two observations:

(1) The *A. pleuropneumoniae* serotype 1 challenge strain not only expressed the 103 kDa cytolysin but, in addition, expressed a serologically distinct 105 kDa cytolysin. This is in accordance with the results of Kamp, E.M, *et al.*, Abstr. *CRWAD* (1990) 1990:270, who described the presence of these two cytolysins in an *A. pleuropneumoniae* serotype 1 strain. Therefore, the lack of protection against heterologous challenge could not only be caused by serotype-specific differences of the 103 kDa cytolysin, but it could also indicate that the activity of one cytolysin is sufficient to allow subsequent colonization by the pathogen.

(2) The *A. pleuropneumoniae* serotype 1 and 7 challenge strains express different 60 kDa proteins. Thus, Southern hybridization of chromosomal DNA from the *A. pleuropneumoniae* serotype 1 challenge strain with the *tfb*A probe did not result in binding under high stringency conditions, and serum raised against the 60 kDa protein did not react strongly with *A. pleuropneumoniae* serotype 1 grown under iron-restricted conditions. The observations concerning the genetic and antigenic differences of the 60 kDa proteins in *A. pleuropneumoniae* serotype 1 and 7 strains, as well as the presence of two different cytolysins in

A. pleuropneumoniae serotype 1 strains, explain th se results. Therefore, thes findings suggest that a vaccine containing at least two serologically and functionally distinct *A. pleuropneumoniae* cytolysins, as well as serotype-specific 60 kDa proteins, might offer cross-protection against clinical symptoms.

Table 2

Mortality, Lung Damage, and Serological Response of Pigs Vaccinated With Recombinant Cytolysin and 60K-protein (Trial 1)

Group	Antigen for Vaccination	Mortality[1]	% Lung Damage[2]	Serotiter[3] Cytolysin	Serotiter[3] 60K-protein	Body Temperature[4]	Clinical Score
	***A. pleuropneumoniae* Challenge Strain: AP 205 (serotype 7)**						
1	None	4/6	17.5 ± 10.4	<200	<200	40.7 ± 0.2	1.75
2	Cytolysin	0/6	14.1 ± 15.5	2400	<200	40.1 ± 0.5	0.625
3	60 kDa Protein	1/6	26.5 ± 26.4	<200	9600	40.4 ± 0.7	1.0
4	Cytolysin and 60 kDa Protein	1/6[5]	3.7 ± 4.5	800	19.200	39.7 ± 0.3	0.25
	***A. pleuropneumoniae* Challenge Strain: AP 37 (serotype 1)**						
5	None	4/6	--	<200	<200	41.4 ± 0.3	2.0
6	Cytolysin	5/6	--	1600	<200	41.8 ± 0.6	1.875
7	60 kDa Protein	4/6	--	<200	19.200	41.4 ± 0.2	1.5
8	Cytolysin and 60 kDa Protein	4/6	--	1600	6400	41.2 ± 0.6	1.75

(see next page for notes)

Table 2 (cont.)

Mortality, Lung Damage, and Serological Response of Pigs Vaccinated With Recombinant Cytolysin and 60K-protein (Trial 1)

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[1] Number of pigs that died or were euthanized *in extremis* over the total in the group.

[2] The lung damage was assessed only for pigs surviving until day 7 after challenge.

[3] The serotiter is the median of the individual titers determined at the date of challenge.

[4] Arithmetic mean body temperature (c) for survivors on the second day after challenge.

[5] The dead pig did not develop a serotiter against the cytolysin.

Table 3

Mortality, Lung Damage, and Serological Response of Pigs Vaccinated With Different Amounts of Recombinant 60 kDa Protein (Trial 2)

A. pleuropneumoniae Challenge Strain	Group	Amount [μg] of Antigen for Vaccination	Mortality[1]	% Lung Damage[1]	Serotiter[2]
AP205 (serotype 7)	1	None	3/6	8.6 ± 6.1	<200
	2	200	1/6	7.0 ± 4.9	51.200
	3	50	1/6	11.9 ± 15.0	25.600
	4	12.5	0/6	7.3 ± 10.2	51.200

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[1] The lung damage was assessed only for pigs surviving until day 7 after challenge.

[2] The serotiter is the median of the individual titers determined at the date of challenge.

Example 7
Cloning of *A. Pleuropneumoniae* Serotype 5 Protective Proteins

A genomic library of *A. pleuropneumoniae* serotype 5 strain AP213 was prepared by partially digesting chromosomal DNA with *Sau*3AI and ligating into the *Bam*HI site of the phage vector λ2001 as described in Example 4. The library was screened under low stringency conditions with an NsiI-KpnI fragment from plasmid pTF205/E1, which encodes the serotype 7 transferrin binding protein (*tfb*A), and with probes from the gene encoding the APP4 protein from serotype 1. The DNA from positive plaques of each type was purified and subcloned into expression vectors as follows. For the rAPP4 gene, recombinant λ2001 DNA was partially digested with *Sau*3AI and ligated into a *Bam*HI-digested pGH432. The ligation mix was transformed into *E. coli* HB101. For the *tfb*A gene, an *Nsi*I fragment from the recombinant phage was subcloned into the *Nsi*I site of plasmid pTF205/E1, in front of the serotype 7 *tfb*A gene. This ligation mix was also transformed into *E. coli* HB101. This construct was trimmed by digesting the plasmid completely with *Bam*HI and partially with *Sau*3AI and religating. This eliminated the *A. pleuropneumoniae* serotype 7 *tfb*A gene and non-coding DNA at the 3′ end of serotype 5 *tfb*A the gene.

The recombinant plasmids expressing the serotype 5 *tfb* gene (pTF213/E6) and the rAPP4 gene (p#4-213-84) were shown to produce polypeptides of approximately 62 kDa and 60 kDa, respectively, which reacted with convalescent serum from an *A. pleuropneumoniae* serotype 5-infected pig. In addition, serum raised against the recombinant tfbA protein reacted specifically with a 62 kDa protein of *A. pleuropneumoniae* serotype 5.

Example 8

The Protective Capacity of Serotype 5 Recombinant Proteins

Serotype 5 r combinant transferrin binding protein and recombinant APP4 were prepared as described in Example 7. Vaccines containing these recombinant proteins were prepared by solubilizing the proteins with guanidine hydrochloride and combining the resultant solution with the adjuvant Emulsigen Plus such that each 2 ml dose contained 25 μg protein and 30% adjuvant, as described in Example 6.

Groups of four pigs were vaccinated as described in Example 6 with the recombinant vaccines and three pigs were immunized with a placebo containing adjuvant only. All animals were boosted three weeks later, and after seven days all pigs were challenged with *A. pleuropneumoniae* serotype 5 strain AP213 (8×10^5 CFU/ml) by aerosol as described in Example 6. Clinical signs of disease were monitored daily for three days post challenge, and one week after challenge. All surviving pigs were euthanized and their lungs were examined for pneumonic lesions.

As shown in Table 4, vaccination with either antigen eliminated mortality associated with *A. pleuropneumoniae* infection and reduced clinical signs of disease.

Table 4

Mortality and Clinical Signs of Disease in Pigs Vaccinated with Recombinant Serotype 5 Transferrin Binding Protein or APP4

Grp	Antigen for Vaccination	Mortality[1]	Clinical Score Day 1	Day 2	Day 3	% Lung Damage[2]
1	Placebo	3/3	1.33	1.58	2.13	ND
2	Tfb[3]	0/4	0.87	0.75	0.38	8.13
3	rAPP4	0/4	1.31	1.25	1.37	18.73

[1] Number of pigs that died or were euthanized *in extremis* over the total in the group.
[2] The lung score was assessed only for pigs surviving until day 7 after challenge.
[3] Transferrin binding protein

Example 9

The Protective Capacity of Serotype 1 APP4 Protein

Serotype 1 recombinant APP4 was prepared as described in Example 7. Vaccines containing the APP4 protein were prepared by solubilizing the protein with guanidine hydrochloride and combining the resultant solution with the adjuvant Amphigen such that each 2 ml dose contained 25 μg protein and 30% adjuvant, as described in Example 6.

Groups of four pigs were vaccinated as described in Example 6 with the recombinant vaccine and three pigs were immunized with a placebo containing adjuvant only. All animals were boosted three weeks later, and after seven days all pigs were challenged with

A. pleuropneumoniae serotype 1 strain AP37 by aerosol as described in Example 6. Clinical signs of disease wer monitored daily for three days post challenge, and one week after challenge. All surviving pigs were uthanized and their lungs were examined for pneumonic lesions.

As shown in Table 5, vaccination with APP4 reduced mortality associated with *A. pleuropneumoniae* infection and reduced clinical signs of disease.

Table 5

Mortality and Clinical Signs of Disease in Pigs Vaccinated with Recombinant Serotype 1 APP4

Group		Mortality[1]	Clinical Score Day 1	Day 2	Day 3
1	Placebo	3/5	2.20	1.00	0.75
2	APP4	1/6	0.58	1.00	0.30

[1] Number of pigs that died or were euthanized *in extremis* over the total in the group.

Thus, subunit vaccines for use against *A. pleuropneumoniae* are disclosed, as are methods of making and using the same. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

CLAIMS

1. A vaccine composition comprising a pharmaceutically acceptable vehicle and a subunit antigen composition, said subunit antigen composition comprising at least one amino acid sequence substantially homologous and functionally equivalent to an immunogenic polypeptide of an *Actinobacillus pleuropneumoniae* protein, said *Actinobacillus pleuropneumoniae* protein selected from the group consisting of an *Actinobacillus pleuropneumoniae* transferrin binding protein, an *Actinobacillus pleuropneumoniae* cytolysin and an *Actinobacillus pleuropneumoniae* APP4.

2. The vaccine composition of claim 1 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* transferrin binding protein, or an immunogenic fragment thereof.

3. The vaccine composition of claim 2 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 7 transferrin binding protein having a molecular mass of approximately 60 kDa, as determined by SDS PAGE.

4. The vaccine composition of claim 3 wherein said transferrin binding protein has an amino acid sequence substantially as depicted in Figure 1.

5. The vaccine composition of claim 2 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 5 transferrin binding protein having a molecular mass of approximately 62 kDa, as determined by SDS PAGE.

6. The vaccine composition of claim 2 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 1 transferrin binding protein having a molecular mass of approximately 65 kDa, as determined by SDS PAGE.

7. The vaccine composition of claim 6 wherein said transferrin binding protein has an amino acid sequence substantially as depicted in Figure 2.

8. The vaccine composition of claim 1 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* cytolysin, or an immunogenic fragment thereof.

9. The vaccine composition of claim 8 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 7 cytolysin having a molecular mass of approximately 103 kDa, as determined by SDS PAGE.

10. The vaccine composition of claim 1 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* APP4.

11. The vaccine composition of claim 10 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 1 APP4.

12. The vaccine composition of claim 10 wherein said immunogenic polypeptide is an *Actinobacillus pleuropneumoniae* serotype 5 APP4.

13. The vaccine composition of claim 1 wherein said subunit antigen composition comprises an *Actinobacillus pleuropneumoniae* transferrin binding

protein, or an immunogenic fragment thereof, and an *Actinobacillus pleuropneumoniae* cytolysin, or an immunogenic fragment thereof.

14. The vaccine composition of claim 13 further comprising an *Actinobacillus pleuropneumoniae* APP4.

15. The vaccine composition of claim 1 further comprising an adjuvant.

16. A nucleotide sequence encoding an *Actinobacillus pleuropneumoniae* transferrin binding protein or a protein substantially homologous and functionally equivalent thereto.

17. The nucleotide sequence of claim 16 wherein said transferrin binding protein comprises an amino acid sequence substantially as depicted in Figure 1.

18. The nucleotide sequence of claim 16 wherein said transferrin binding protein comprises an amino acid sequence substantially as depicted in Figure 2.

19. The nucleotide sequence of claim 16 wherein said transferrin binding protein comprises an amino acid sequence substantially as encoded by the nucleotide sequence present in recombinant plasmid pTF213/E6.

20. A nucleotide sequence encoding an *Actinobacillus pleuropneumoniae* APP4 protein or a protein

substantially homologous and functionally equivalent thereto.

21. The nucleotide sequence of claim 20 wherein said APP4 protein comprises an amino acid sequence substantially as encoded by the nucleotide sequence present in recombinant plasmid prAPP4.

22. The nucleotide sequence of claim 20 wherein said APP4 protein comprises an amino acid sequence substantially as encoded by the nucleotide sequence present in recombinant plasmid p#4-213-84.

23. A DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* transferrin binding protein; and

(b) control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

24. The DNA construct of claim 23 wherein said DNA coding sequence encodes at least one epitope of an *Actinobacillus pleuropneumoniae* serotype 7 60 kDa transferrin binding protein.

25. The DNA construct of claim 23 wherein said DNA coding sequence encodes at least one epitope of an *Actinobacillus pleuropneumoniae* serotype 5 62 kDa transferrin binding protein.

26. The DNA construct f claim 23 wherein said DNA coding sequence ncodes at least one epitope of an *Actinobacillus pleuropneumoniae* serotyp 1 65 kDa transferrin binding protein.

27. A DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* cytolysin; and

(b) control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

28. The DNA construct of claim 27 wherein said DNA coding sequence encodes at least one epitope of an *Actinobacillus pleuropneumoniae* serotype 7 103 kDa cytolysin.

29. A DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of an *Actinobacillus pleuropneumoniae* APP4; and

(b) control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

30. The DNA construct of claim 29 wherein said DNA coding sequence encodes at least one epitope of an *Actinobacillus pleuropneumoniae* serotype 1 APP4.

31. The DNA construct of claim 29 wherein said DNA coding sequence encodes at 1 ast one epitope of an *Actinobacillus pleuropneumoniae* serotype 5 APP4.

32. A host cell stably transformed by a DNA construct according to any of claims 23-31.

33. A method of producing a recombinant polypeptide comprising:

(a) providing a population of host cells according to claim 32; and

(b) growing said population of cells under conditions whereby the polypeptide encoded by said expression cassette is expressed.

34. A method of treating or preventing pneumonia in swine comprising administering to said swine a therapeutically effective amount of a vaccine composition according to any of claims 1-15.

35. Isolated and purified *Actinobacillus pleuropneumoniae* serotype 7 60 kDa transferrin binding protein.

36. Isolated and purified *Actinobacillus pleuropneumoniae* serotype 5 62 kDa transferrin binding protein.

37. Isolated and purified *Actinobacillus pleuropneumoniae* serotype 1 65 kDa transferrin binding protein.

38. Isolated and purified *Actinobacillus pleuropneumoniae* serotype 1 APP4.

39. Isolated and purified *Actinobacillus pleuropneumoniae* serotyp 5 APP4.

```
        10         20         30         40         50         60         70         80         90        100        110        120
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ACAATGCCAATATTAACCCAATCTATTCCACTTGAATTACCAACCTCCAGTATTGAGAAAAAAGATGAGCCAAAAGATATCTTCAGAGTGGCGATTAATCCTACGGGCATTTATTTAGGCG
TGTTACGGTTATAATTGGGTTAGATAAGGTGAACTTAATGGTTGGAGGTCATAACTCTTTTTTCTACTCGGTTTTCTATAGAAGTCTCACCGCTAATTAGGATGCCCGTAAATAAATCCGC

       130        140        150        160        170        180        190        200        210        220        230        240
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AGAAGCTAGTGAATGAAGAAGAATTAAAACAATCTTTTCTGACAAAATTTCAGGAAAATAAAAATACCGTTATTGCTATTTCTGCGGATATTTCCGTGGAATATCAACATATCGTGAAAGT
TCTTCGATCACTTACTTCTTCTTAATTTTGTTAGAAAAGACTGTTTTAAAGTCCTTTTATTTTTATGGCAATAACGATAAAGACGCCTATAAAGGCACCTTATAGTTGTATAGCACTTTCA

       250        260        270        280        290        300        310        320        330         340          350
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *     *     *     *
CCTTGAATTAGCTCAAAACGTCGGGCTAACGAAAATAGGCTTTGTGACTCACCTAGTAAATAAAAGCAGAAATTTTATATTGGAGGCAAT ATG CAT TTT AAA CTT AAT CCC
GGAACTTAATCGAGTTTTGCAGCCCGATTGCTTTTATCCGAAACACTGAGTGGATCATTTATTTTCGTCTTTAAAATATAACCTCCGTTA TAC GTA AAA TTT GAA TTA GGG
                                                                                            Met His Phe Lys Leu Asn Pro>
                                                                                            ____OPEN READING FRAME_a___>

       360          370          380          390          400          410          420          430          440
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TAT GCG TTA GCG TTT ACT TCG CTG TTT CTT GTC GCT TGT TCT GGC GGA AAA GGA AGT TTT GAT TTA GAA GAT GTC CGG CCT AAT AAG ACA
ATA CGC AAT CGC AAA TGA AGC GAC AAA GAA CAG CGA ACA AGA CCG CCT TTT CCT TCA AAA CTA AAT CTT CTA CAG GCC GGA TTA TTC TGT
Tyr Ala Leu Ala Phe Thr Ser Leu Phe Leu Val Ala Cys Ser Gly Gly Lys Gly Ser Phe Asp Leu Glu Asp Val Arg Pro Asn Lys Thr>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 1

```
        450       460       470       480       490       500       510       520       530
 #       *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ACA GGC GTG TCT AAA GAG GAG TAC AAG GAT GTA GAA ACA GCC AAG AAA GAA AAA GAA CAG TTA GGG GAA TTA ATG GAA CCT GCT TTG GGG
TGT CCG CAC AGA TTT CTC CTC ATG TTC CTA CAT CTT TGT CGG TTC TTT CTT TTT CTT GTC AAT CCC CTT AAT TAC CTT GGA CGA AAC CCC
Thr Gly Val Ser Lys Glu Glu Tyr Lys Asp Val Glu Thr Ala Lys Lys Glu Lys Glu Gln Leu Gly Glu Leu Met Glu Pro Ala Leu Gly>
___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

        540       550       560       570       580       590       600       610       620
 #       *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TAT GTT GTA AAA GTT CCG GTG AGT TCT TTT GAA AAT AAG AAA GTT GAT ATT TCA GAT ATA GAA GTG ATT ACG AAC GGA AAT TTA GAC GAT
ATA CAA CAT TTT CAA GGC CAC TCA AGA AAA CTT TTA TTC TTT CAA CTA TAA AGT CTA TAT CTT CAC TAA TGC TTG CCT TTA AAT CTG CTA
Tyr Val Val Lys Val Pro Val Ser Ser Phe Glu Asn Lys Lys Val Asp Ile Ser Asp Ile Glu Val Ile Thr Asn Gly Asn Leu Asp Asp>
___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

        630       640       650       660       670       680       690       700       710
 #       *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTG CCG TAC AAG GCA AAT TCA TCT AAA TAT AAC TAT CCA GAT ATA AAA ACA AAA GAT TCT TCT CTT CAG TAC GTT CGC TCA GGA TAT GTT
CAC GGC ATG TTC CGT TTA AGT AGA TTT ATA TTG ATA GGT CTA TAT TTT TGT TTT CTA AGA AGA GAA GTC ATG CAA GCG AGT CCT ATA CAA
Val Pro Tyr Lys Ala Asn Ser Ser Lys Tyr Asn Tyr Pro Asp Ile Lys Thr Lys Asp Ser Ser Leu Gln Tyr Val Arg Ser Gly Tyr Val>
___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

        720       730       740       750       760       770       780       790       800
 #       *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ATT GAT GGG GAA CAC TCT GGT TCT AAT GAA AAG GGA TAT GTG TAT TAT AAA GGT AAT TCA CCT GCA AAA GAA TTA CCC GTT AAT CAG CTT
TAA CTA CCC CTT GTG AGA CCA AGA TTA CTT TTC CCT ATA CAC ATA ATA TTT CCA TTA AGT GGA CGT TTT CTT AAT GGG CAA TTA GTC GAA
Ile Asp Gly Glu His Ser Gly Ser Asn Glu Lys Gly Tyr Val Tyr Tyr Lys Gly Asn Ser Pro Ala Lys Glu Leu Pro Val Asn Gln Leu>
___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>
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FIGURE 1 CONT'D

```
     810        820        830         840        850        860         870        880        890
 *     *    *     *     *    *     *     *    *     *    *     *     *    *     *     *     *
TTA ACT TAT ACA GGA AGT TGG GAT TTT ACT TCC AAT GCG AAT TTA AAT AAT GAA GAG GGA AGA CCT AAT TAT TTA AAC GAC GAT TAT TAT
AAT TGA ATA TGT CCT TCA ACC CTA AAA TGA AGG TTA CGC TTA AAT TTA TTA CTT CTC CCT TCT GGA TTA ATA AAT TTG CTG CTA ATA ATA
Leu Thr Tyr Thr Gly Ser Trp Asp Phe Thr Ser Asn Ala Asn Leu Asn Asn Glu Glu Gly Arg Pro Asn Tyr Leu Asn Asp Asp Tyr Tyr>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

     900        910        920         930        940        950         960        970        980
 *     *    *     *     *    *     *     *    *     *    *     *     *    *     *     *     *
ACT AAA TTT ATA GGT AAA CGG GTG GGC TTG GTT TCG GGA GAT GCG AAA CCT GCA AAG CAT AAA TAC ACT AGC CAG TTT GAA GTT GAT TTT
TGA TTT AAA TAT CCA TTT GCC CAC CCG AAC CAA AGC CCT CTA CGC TTT GGA CGT TTC GTA TTT ATG TGA TCG GTC AAA CTT CAA CTA AAA
Thr Lys Phe Ile Gly Lys Arg Val Gly Leu Val Ser Gly Asp Ala Lys Pro Ala Lys His Lys Tyr Thr Ser Gln Phe Glu Val Asp Phe>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

     990       1000       1010        1020       1030       1040        1050       1060       1070
 *     *    *     *     *    *     *     *    *     *    *     *     *    *     *     *     *
GCA ACT AAA AAA ATG ACA GGT AAA TTA TCC GAT AAA GAG AAA ACG ATT TAT ACA GTC AAT GCT GAT ATT AGA GGC AAT CGT TTT ACG GGG
CGT TGA TTT TTT TAC TGT CCA TTT AAT AGG CTA TTT CTC TTT TGC TAA ATA TGT CAG TTA CGA CTA TAA TCT CCG TTA GCA AAA TGC CCC
Ala Thr Lys Lys Met Thr Gly Lys Leu Ser Asp Lys Glu Lys Thr Ile Tyr Thr Val Asn Ala Asp Ile Arg Gly Asn Arg Phe Thr Gly>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1080       1090       1100        1110       1120       1130        1140       1150       1160
 *     *    *     *     *    *     *     *    *     *    *     *     *    *     *     *     *
GCT GCT ACA GCG AGT GAT AAA AAT AAA GGG AAA GGC GAA TCA TAT AAC TTC TTT AGT GCC GAT TCT CAG TCT TTA GAA GGC GGC TTC TAT
CGA CGA TGT CGC TCA CTA TTT TTA TTT CCC TTT CCG CTT AGT ATA TTG AAG AAA TCA CGG CTA AGA GTC AGA AAT CTT CCG CCG AAG ATA
Ala Ala Thr Ala Ser Asp Lys Asn Lys Gly Lys Gly Glu Ser Tyr Asn Phe Phe Ser Ala Asp Ser Gln Ser Leu Glu Gly Gly Phe Tyr>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 1 CONT'D

```
      1170        1180        1190          1200        1210        1220          1230        1240        1250
 *      *    *      *      *     *      *      *     *      *      *     *      *      *     *      *      *     *
GGT CCA AAA GCA GAA GAA ATG GCA GGG AAA TTT GTA GCT AAC GAC AAA TCT CTT TTT GCC GTT TTT TCA GCA AAA CAC AAT GGC TCT AAT
CCA GGT TTT CGT CTT CTT TAC CGT CCC TTT AAA CAT CGA TTG CTG TTT AGA GAA AAA CGG CAA AAA AGT CGT TTT GTG TTA CCG AGA TTA
Gly Pro Lys Ala Glu Glu Met Ala Gly Lys Phe Val Ala Asn Asp Lys Ser Leu Phe Ala Val Phe Ser Ala Lys His Asn Gly Ser Asn>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME__a___a___a___a___a___a___a___a___a___a___a___a___>
```

```
      1260        1270        1280          1290        1300        1310          1320        1330        1340
 *      *    *      *      *     *      *      *     *      *      *     *      *      *     *      *      *     *
GTT AAC ACC GTT CGG ATT ATT GAT GCC TCA AAA ATT GAT TTA ACT AAT TTC AGC ATT TCA GAA CTT AAC AAT TTT GGT GAT GCT TCC GTT
CAA TTG TGG CAA GCC TAA TAA CTA CGG AGT TTT TAA CTA AAT TGA TTA AAG TCG TAA AGT CTT GAA TTG TTA AAA CCA CTA CGA AGG CAA
Val Asn Thr Val Arg Ile Ile Asp Ala Ser Lys Ile Asp Leu Thr Asn Phe Ser Ile Ser Glu Leu Asn Asn Phe Gly Asp Ala Ser Val>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME__a___a___a___a___a___a___a___a___a___a___a___a___>
```

```
      1350        1360        1370          1380        1390        1400          1410        1420        1430
 *      *    *      *      *     *      *      *     *      *      *     *      *      *     *      *      *     *
TTA ATT ATT GAT GGG AAA AAA ATA AAG CTA GCT GGT AGC GGG TTT ACA AAT AAG CAC ACT ATT GAA ATC AAT GGC AAA ACA ATG GTA GCC
AAT TAA TAA CTA CCC TTT TTT TAT TTC GAT CGA CCA TCG CCC AAA TGT TTA TTC GTG TGA TAA CTT TAG TTA CCG TTT TGT TAC CAT CGG
Leu Ile Ile Asp Gly Lys Lys Ile Lys Leu Ala Gly Ser Gly Phe Thr Asn Lys His Thr Ile Glu Ile Asn Gly Lys Thr Met Val Ala>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME__a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 1 CONT'D

```
    1440      1450      1460      1470      1480      1490      1500      1510      1520
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTA GCC TGC TGT AGT AAT CTG GAA TAT ATG AAG TTT GGT CAA TTA TGG CAA CAA GCA GAG GGC GGA AAA CCC GAG AAT AAT AGT TTA TTC
CAT CGG ACG ACA TCA TTA GAC CTT ATA TAC TTC AAA CCA GTT AAT ACC GTT GTT CGT CTC CCG CCT TTT GGG CTC TTA TTA TCA AAT AAG
Val Ala Cys Cys Ser Asn Leu Glu Tyr Met Lys Phe Gly Gln Leu Trp Gln Gln Ala Glu Gly Gly Lys Pro Glu Asn Asn Ser Leu Phe>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1530      1540      1550      1560      1570      1580      1590      1600      1610
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTA CAA GGC GAA CGT ACC GCA ACA GAT AAG ATG CCA AAA GGC GGA AAC TAT AAA TAT ATT GGT ACT TGG GAT GCT CAG GTT TCA AAA GAA
GAT GTT CCG CTT GCA TGG CGT TGT CTA TTC TAC GGT TTT CCG CCT TTG ATA TTT ATA TAA CCA TGA ACC CTA CGA GTC CAA AGT TTT CTT
Leu Gln Gly Glu Arg Thr Ala Thr Asp Lys Met Pro Lys Gly Gly Asn Tyr Lys Tyr Ile Gly Thr Trp Asp Ala Gln Val Ser Lys Glu>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1620      1630      1640      1650      1660      1670      1680      1690      1700
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAT AAC TGG GTT GCT ACG GCA GAT GAT GAT AGA AAA GCT GGC TAT CGG ACA GAA TTT GAT GTT GAT TTT GGC AAC AAA AAT TTA AGT GGT
TTA TTG ACC CAA CGA TGC CGT CTA CTA CTA TCT TTT CGA CCG ATA GCC TGT CTT AAA CTA CAA CTA AAA CCG TTG TTT TTA AAT TCA CCA
Asn Asn Trp Val Ala Thr Ala Asp Asp Asp Arg Lys Ala Gly Tyr Arg Thr Glu Phe Asp Val Asp Phe Gly Asn Lys Asn Leu Ser Gly>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1710      1720      1730      1740      1750      1760      1770      1780      1790
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAG TTA TTT GAT AAA AAC GGT GTA AAT CCT GTG TTT ACC GTA GAT GCA AAA ATT GAT GGT AAT GGT TTT ACT GGC AAA GCT AAA ACC TCA
TTC AAT AAA CTA TTT TTG CCA CAT TTA GGA CAC AAA TGG CAT CTA CGT TTT TAA CTA CCA TTA CCA AAA TGA CCG TTT CGA TTT TGG AGT
Lys Leu Phe Asp Lys Asn Gly Val Asn Pro Val Phe Thr Val Asp Ala Lys Ile Asp Gly Asn Gly Phe Thr Gly Lys Ala Lys Thr Ser>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 1 CONT'D

```
    1800      1810      1820      1830      1840      1850      1860      1870      1880.
GAT GAA GGC TTC GCT CTA GAT TCA GGT AGT TCA CGT TAT GAG AAT GTG AAA TTT AAC GAT GTA GCA GTT AGT GGT GGC TTC TAT GGT CCA
CTA CTT CCG AAG CGA GAT CTA AGT CCA TCA AGT GCA ATA CTC TTA CAC TTT AAA TTG CTA CAT CGT CAA TCA CCA CCG AAG ATA CCA GGT
Asp Glu Gly Phe Ala Leu Asp Ser Gly Ser Ser Arg Tyr Glu Asn Val Lys Phe Asn Asp Val Ala Val Ser Gly Gly Phe Tyr Gly Pro>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1890      1900      1910      1920      1930      1940      1950      1960      1970
ACG GCA GCA GAG CTT GGC GGA CAA TTC CAC CAT AAA TCA GAA AAT GGC AGT GTA GGT GCT GTC TTT GGT GCA AAA CAA CAA GTA AAA AAA
TGC CGT CGT CTC GAA CCG CCT GTT AAG GTG GTA TTT AGT CTT TTA CCG TCA CAT CCA CGA CAG AAA CCA CGT TTT GTT GTT CAT TTT TTT
Thr Ala Ala Glu Leu Gly Gly Gln Phe His His Lys Ser Glu Asn Gly Ser Val Gly Ala Val Phe Gly Ala Lys Gln Gln Val Lys Lys>
___a___a___a___a___a___a___a___a___a___a___a___a__OPEN READING FRAME___a___a___a___a___a___a___a___a___a___a___a___a___>

    1980      1990      2000      2010      2020      2030      2040      2050      2060      2070      2080      2090
TAA TAAGGAATTTGCAATGAAAAATAAATTAAATCTGATTAGCCTTGCTCTGCTTAGCCTCTTTGCCGTACAAAGCTATGCAGAACAAGCGGTGCAATTGAACGATGTTTATGTCACAGG
ATT ATTCCTTAAACGTTACTTTTTATTTAATTTAGACTAATCGGAACGAGACGAATCGGAGAAACGGCATGTTTCGATACGTCTTGTTCGCCACGTTAACTTGCTACAAATACAGTGTCC
End>
___>
```

FIGURE 1 CONT'D

```
2100       2110       2120       2130       2140       2150       2160       2170       2180       2190       2200       2210
*    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
TACCAAAAAGAAAGCACATAAAAAAGAGAACGAAGTGACAGGCTTAGGGAAAGTAGTGAAAACACCAGATTCTCTTAGTAAGGAGCAAGTGTTAGGAATGCGAGATCTGACTCGCTACGAT
ATGGTTTTTCTTTCGTGTATTTTTTCTCTTGCTTCACTGTCCGAATCCCTTTCATCACTTTTGTGGTCTAAGAGAATCATTCCTCGTTCACAATCCTTACGCTCTAGACTGAGCGATGCTA

2220       2230       2240       2250       2260       2270       2280       2290       2300       2310       2320       2330
*    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
CCGGGTATTTCTGTAGTAGAGCAAGGACGAGGTGCAACGACAGGCTACTCAATTCGTGGGGTAGATCGTAATCGTGTGGGCTTGGCATTAGACGGTTTGCCACAGATTCAATCCTATGTAA
GGCCCATAAAGACATCATCTCGTTCCTGCTCCACGTTGCTGTCCGATGAGTTAAGCACCCCATCTAGCATTAGCACACCCGAACCGTAATCTGCCAAACGGTGTCTAAGTTAGGATACATT

2340       2350       2360       2370       2380       2390       2400       2410       2420       2430       2440       2450
*    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
GTCAATATTCACGTTCCTCAAGCGGTGCCATTAATGAAATAGAATACGAAAATCTGCGTTCGATCCAAATTAGTAAAGGAGCTAGTTCTTCTGAGTTTGGCAGTGGCTCGCTAGGCGGTTC
CAGTTATAAGTGCAAGGAGTTCGCCACGGTAATTACTTTATCTTATGCTTTTAGACGCAAGCTAGGTTTAATCATTTCCTCGATCAAGAAGACTCAAACCGTCACCGAGCGATCCGCCAAG

2460       2470       2480       2490       2500       2510       2520       2530       2540       2550       2560       2570
*    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
GGTGCAATTCCGTACCAAAGAGGTAAGCGACATTATTAAGCCAGGGCAATCTTGGGGACTAGATACCAAAAGTGCCTACAGCAGCAAAAATCAACAATGGTTAAACTCACTTGCTTTTGCG
CCACGTTAAGGCATGGTTTCTCCATTCGCTGTAATAATTCGGTCCCGTTAGAACCCCTGATCTATGGTTTTCACGGATGTCGTCGTTTTTAGTTGTTACCAATTTGAGTGAACGAAAACGC

2580       2590       2600       2610       2620       2630       2640       2650       2660       2670       2680       2690
*    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
GGTACTCACAATGGCTTTGAGTCTCTTGTGATTTACACTCACCGTGATGGTAAGGAAACGAAAGCTCATAAGGATGCAGAAAGCCGTTCTAAGAGTATTCAGAGAGTGGATCTAAGCTT
CCATGAGTGTTACCGAAACTCAGAGAACACTAAATGTGAGTGGCACTACCATTCCTTTGCTTTCGAGTATTCCTACGTCTTTCGGCAAGATTCTCATAAGTCTCTCACCTAGATTCGAA
```

FIGURE 1 CONT'D

```
         10          20          30          40          50          60          70          80          90
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ATG CAT TTT AAA CTT AAT CCC TAT GCG TTA GCG TTT ACT TCG CTG TTT CTT GTC GCT TGT TCT GGC GGA AAA GGA AGT TTT GAT TTA GAA
TAC GTA AAA TTT GAA TTA GGG ATA CGC AAT CGC AAA TGA AGC GAC AAA GAA CAG CGA ACA AGA CCG CCT TTT CCT TCA AAA CTA AAT CTT
Met His Phe Lys Leu Asn Pro Tyr Ala Leu Ala Phe Thr Ser Leu Phe Leu Val Ala Cys Ser Gly Gly Lys Gly Ser Phe Asp Leu Glu>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

        100         110         120         130         140         150         160         170         180
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAT GTC CGG CCA AAT CAA ACT GCA AAA GCA GAA AAA GCA ACA ACC TCT TAT CAA GAT GAG GAA ACG AAG AAA AAG ACA AAG GAA GAA TTA
CTA CAG GCC GGT TTA GTT TGA CGT TTT CGT CTT TTT CGT TGT TGG AGA ATA GTT CTA CTC CTT TGC TTC TTT TTC TGT TTC CTT CTT AAT
Asp Val Arg Pro Asn Gln Thr Ala Lys Ala Glu Lys Ala Thr Thr Ser Tyr Gln Asp Glu Glu Thr Lys Lys Lys Thr Lys Glu Glu Leu>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

        190         200         210         220         230         240         250         260         270
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAT AAG TTG ATG GAG CCT GCT TTG GGG TAT GAA ACT CAA ATT TTA CGG CGA AAT AAG GCT CCT AAA ACA GAA ACA GGA GAG AAA AGG AAT
CTA TTC AAC TAC CTC GGA CGA AAC CCC ATA CTT TGA GTT TAA AAT GCC GCT TTA TTC CGA GGA TTT TGT CTT TGT CCT CTC TTT TCC TTA
Asp Lys Leu Met Glu Pro Ala Leu Gly Tyr Glu Thr Gln Ile Leu Arg Arg Asn Lys Ala Pro Lys Thr Glu Thr Gly Glu Lys Arg Asn>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

        280         290         300         310         320         330         340         350         360
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAG AGA GTT GTT GAG TTA TCC GAA GAT AAA ATT ACG AAA TTA TAC CAA GAG AGT GTA GAA ATA ATC CCT CAT TTA GAT GAG CTA AAT GGA
CTC TCT CAA CAA CTC AAT AGG CTT CTA TTT TAA TGC TTT AAT ATG GTT CTC TCA CAT CTT TAT TAG GGA GTA AAT CTA CTC GAT TTA CCT
Glu Arg Val Val Glu Leu Ser Glu Asp Lys Ile Thr Lys Leu Tyr Gln Glu Ser Val Glu Ile Ile Pro His Leu Asp Glu Leu Asn Gly>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a---a--->
```

FIGURE 2

```
       370       380       390       400       410       420       430       440       450
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAA ACA ACG AGC AAT GAT GTT TAT CAT TCT CAC GAT AGT AAA AGG CTT GAT AAG AAT AGA GAT CTC AAA TAT GTT CGT TCA GGT TAT GTT
TTT TGT TGC TCG TTA CTA CAA ATA GTA AGA GTG CTA TCA TTT TCC GAA CTA TTC TTA TCT CTA GAG TTT ATA CAA GCA AGT CCA ATA CAA
Lys Thr Thr Ser Asn Asp Val Tyr His Ser His Asp Ser Lys Arg Leu Asp Lys Asn Arg Asp Leu Lys Tyr Val Arg Ser Gly Tyr Val>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

       460       470       480       490       500       510       520       530       540
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TAT GAT GGG TCT TTC AAT GAA ATA CGA CGA AAT GAC TCA GGA TTC CAT GTT TTT AAA CAG GGT ATA GAT GGC TAT GTC TAT TAC CTT GGA
ATA CTA CCC AGA AAG TTA CTT TAT GCT GCT TTA CTG AGT CCT AAG GTA CAA AAA TTT GTC CCA TAT CTA CCG ATA CAG ATA ATG GAA CCT
Tyr Asp Gly Ser Phe Asn Glu Ile Arg Arg Asn Asp Ser Gly Phe His Val Phe Lys Gln Gly Ile Asp Gly Tyr Val Tyr Tyr Leu Gly>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

       550       560       570       580       590       600       610       620       630
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTT ACT CCA TCA AAA GAG TTA CCA AAA GGA AAA GTC ATA AGT TAT AAA GGT ACT TGG GAT TTT GTA AGT AAC ATC AAT TTA GAG CGT GAA
CAA TGA GGT AGT TTT CTC AAT GGT TTT CCT TTT CAG TAT TCA ATA TTT CCA TGA ACC CTA AAA CAT TCA TTG TAG TTA AAT CTC GCA CTT
Val Thr Pro Ser Lys Glu Leu Pro Lys Gly Lys Val Ile Ser Tyr Lys Gly Thr Trp Asp Phe Val Ser Asn Ile Asn Leu Glu Arg Glu>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

       640       650       660       670       680       690       700       710       720
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ATA GAT GGA TTC GAC ACT TCA GGT GAT GGT AAA AAT GTA TCT GCA ACA TCT ATT ACA GAA ACT GTC AAT CGA GAT CAT AAA GTT GGT GAA
TAT CTA CCT AAG CTG TGA AGT CCA CTA CCA TTT TTA CAT AGA CGT TGT AGA TAA TGT CTT TGA CAG TTA GCT CTA GTA TTT CAA CCA CTT
Ile Asp Gly Phe Asp Thr Ser Gly Asp Gly Lys Asn Val Ser Ala Thr Ser Ile Thr Glu Thr Val Asn Arg Asp His Lys Val Gly Glu>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->
```

FIGURE 2 CONT'D

```
       730       740       750       760       770       780       790       800       810
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAA CTA GGT GAT AAT GAA GTT AAA GGG GTA GCT CAT TCT AGT GAA TTT GCA GTA GAT TTT GAT AAC|AAA AAA TTG ACA GGT AGT TTA TAT
TTT GAT CCA CTA TTA CTT CAA TTT CCC CAT CGA GTA AGA TCA CTT AAA CGT CAT CTA AAA CTA TTG|TTT TTT AAC TGT CCA TCA AAT ATA
Lys Leu Gly Asp Asn Glu Val Lys Gly Val Ala His Ser Ser Glu Phe Ala Val Asp Phe Asp Asn|Lys Lys Leu Thr Gly Ser Leu Tyr>
___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

       820       830       840       850       860       870       880       890       900
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGT AAT GGT TAT ATC AAC AGA AAT AAA GCG CAA GAA GTA ACG AAA CGC TAT AGC ATT GAA GCT GAT ATT GCA GGC AAC CGT TTT AGG GGA
GCA TTA CCA ATA TAG TTG TCT TTA TTT CGC GTT CTT CAT TGC TTT GCG ATA TCG TAA CTT CGA CTA TAA CGT CCG TTG GCA AAA TCC CCT
Arg Asn Gly Tyr Ile Asn Arg Asn Lys Ala Gln Glu Val Thr Lys Arg Tyr Ser Ile Glu Ala Asp Ile Ala Gly Asn Arg Phe Arg Gly>
___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

       910       920       930       940       950       960       970       980       990
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAA GCC AAA GCA GAA AAA GCA GGT GAT CCG ATC TTT ACT GAT TCA AAT TAT CTT GAA GGG GGA TTC TAT GGT CCT AAA GCT GAA GAA ATG
TTT CGG TTT CGT CTT TTT CGT CCA CTA GGC TAG AAA TGA CTA AGT TTA ATA GAA CTT CCC CCT AAG ATA CCA GGA TTT CGA CTT CTT TAC
Lys Ala Lys Ala Glu Lys Ala Gly Asp Pro Ile Phe Thr Asp Ser Asn Tyr Leu Glu Gly Gly Phe Tyr Gly Pro Lys Ala Glu Glu Met>
___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

       1000      1010      1020      1030      1040      1050      1060      1070      1080
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCA GGG AAG TTT TTC ACA AAT AAT AAA TCT CTC TTT GCA GTA TTT GCA GCT AAA AGT GAA AAC GGC GAG ACG ACC ACA GAA CGA ATC ATT
CGT CCC TTC AAA AAG TGT TTA TTA TTT AGA GAG AAA CGT CAT AAA CGT CGA TTT TCA CTT TTG CCG CTC TGC TGG TGT CTT GCT TAG TAA
Ala Gly Lys Phe Phe Thr Asn Asn Lys Ser Leu Phe Ala Val Phe Ala Ala Lys Ser Glu Asn Gly Glu Thr Thr Thr Glu Arg Ile Ile>
___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 2 CONT'D

```
        1090        1100        1110        1120        1130        1140        1150        1160        1170
   *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAT GCA ACT AAA ATT GAT TTA ACC CAA TTT AAT GCT AAA GAA CTC AAC AAT TTT GGT GAT GCC TCT GTT TTA ATT ATT GAT GGA CAA AAA
CTA CGT TGA TTT TAA CTA AAT TGG GTT AAA TTA CGA TTT CTT GAG TTG TTA AAA CCA CTA CGG AGA CAA AAT TAA TAA CTA CCT GTT TTT
Asp Ala Thr Lys Ile Asp Leu Thr Gln Phe Asn Ala Lys Glu Leu Asn Asn Phe Gly Asp Ala Ser Val Leu Ile Ile Asp Gly Gln Lys>
___a___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

        1180        1190        1200        1210        1220        1230        1240        1250        1260
   *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ATA GAT CTA GCA GGT GTC AAT TTT AAA AAT AGT AAA ACG GTT GAA ATC AAC GGC AAA ACA ATG GTA GCC GTA GCT TGC TGT AGT AAT CTG
TAT CTA GAT CGT CCA CAG TTA AAA TTT TTA TCA TTT TGC CAA CTT TAG TTG CCG TTT TGT TAC CAT CGG CAT CGA ACG ACA TCA TTA GAC
Ile Asp Leu Ala Gly Val Asn Phe Lys Asn Ser Lys Thr Val Glu Ile Asn Gly Lys Thr Met Val Ala Val Ala Cys Cys Ser Asn Leu>
___a___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

        1270        1280        1290        1300        1310        1320        1330        1340        1350
   *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAA TAT ATG AAA TTT GGT CAA TTG TGG CAA AAA GAG GGC AAA CAA CAA GTT AAA GAT AAT AGT TTA TTC CTA CAA GGT GAA CGT ACT GCA
CTT ATA TAC TTT AAA CCA GTT AAC ACC GTT TTT CTC CCG TTT GTT GTT CAA TTT CTA TTA TCA AAT AAG GAT GTT CCA CTT GCA TGA CGT
Glu Tyr Met Lys Phe Gly Gln Leu Trp Gln Lys Glu Gly Lys Gln Gln Val Lys Asp Asn Ser Leu Phe Leu Gln Gly Glu Arg Thr Ala>
___a___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>
```

FIGURE 2 CONT'D

```
        1360        1370        1380        1390        1400        1410        1420        1430        1440
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ACG GAT AAA ATG CCC GCA GGA GGT AAC TAT AAG TAT GTT GGA ACT TGG GAT GCA CTC GTA TCT AAA GGG ACG AAC TGG ATA GCG GAA GCA
TGC CTA TTT TAC GGG CGT CCT CCA TTG ATA TTC ATA CAA CCT TGA ACC CTA CGT GAG CAT AGA TTT CCC TGC TTG ACC TAT CGC CTT CGT
Thr Asp Lys Met Pro Ala Gly Gly Asn Tyr Lys Tyr Val Gly Thr Trp Asp Ala Leu Val Ser Lys Gly Thr Asn Trp Ile Ala Glu Ala>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

        1450        1460        1470        1480        1490        1500        1510        1520        1530
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAT AAT AAT CGA GAA TCG GGC TAT CGC ACT GAA TTT GAT GTT AAT TTT AGT GAT AAA AAA GTA AAC GGT AAG TTA TTT GAT AAA GGC GGT
CTA TTA TTA GCT CTT AGC CCG ATA GCG TGA CTT AAA CTA CAA TTA AAA TCA CTA TTT TTT CAT TTG CCA TTC AAT AAA CTA TTT CCG CCA
Asp Asn Asn Arg Glu Ser Gly Tyr Arg Thr Glu Phe Asp Val Asn Phe Ser Asp Lys Lys Val Asn Gly Lys Leu Phe Asp Lys Gly Gly>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->

        1540        1550        1560        1570        1580        1590        1600        1610        1620
    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GTA AAT CCT GTA TTT ACC GTA GAT GCG ACA ATT AAT GGT AAT GGC TTT ATC GGC AGT GCG AAA ACC TCT GAT AGT GGC TTT GCT TTA GAT
CAT TTA GGA CAT AAA TGG CAT CTA CGC TGT TAA TTA CCA TTA CCG AAA TAG CCG TCA CGC TTT TGG AGA CTA TCA CCG AAA CGA AAT CTA
Val Asn Pro Val Phe Thr Val Asp Ala Thr Ile Asn Gly Asn Gly Phe Ile Gly Ser Ala Lys Thr Ser Asp Ser Gly Phe Ala Leu Asp>
---a---a---a---a---a---a---a---a---a---a---a---a---a---a-------a---a---a---a---a---a---a---a---a---a---a---a---a---a--->
```

FIGURE 2 CONT'D

```
        1630      1640      1650      1660      1670      1680      1690      1700      1710
           *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCA GGC TCT AGC CAA CAC GGA AAT GCG GTA TTT AGT GAT ATA AAA GTC AAT GGT GGC TTC TAT GGT CCA ACC GCT GGA GAA CTT GGC GGA
CGT CCG AGA TCG GTT GTG CCT TTA CGC CAT AAA TCA CTA TAT TTT CAG TTA CCA CCG AAG ATA CCA GGT TGG CGA CCT CTT GAA CCG CCT
Ala Gly Ser Ser Gln His Gly Asn Ala Val Phe Ser Asp Ile Lys Val Asn Gly Gly Phe Tyr Gly Pro Thr Ala Gly Glu Leu Gly Gly>
___a___a___a___a___a___a___a___a___a___a___a___a___a___a_______a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

        1720      1730      1740      1750      1760      1770      1780      1790      1800
           *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CAA TTC CAT CAT AAA TCA GAC AAT GGC AGT GTT GGN GCT GTC TTT GGT GCA AAA CGA CAA ATA GAA AAA TAATAAGGAATTTGCTATGAAAAATAAAT
GTT AAG GTA GTA TTT AGT CTG TTA CCG TCA CAA CCN CGA CAG AAA CCA CGT TTT GCT GTT TAT CTT TTT ATTATTCCTTAAACGATACTTTTTATTTA
Gln Phe His His Lys Ser Asp Asn Gly Ser Val Gly Ala Val Phe Gly Ala Lys Arg Gln Ile Glu Lys>
___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___a___>

1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TAAATCTGATTAGCCTTGCTCTTCTTAGCCTATTTGCCGTACAAAGCTATGCAGAACAAGCGGTACAATTAAATGATGTTTATGTCACAGGTACC
ATTTAGACTAATCGGAACGAGAAGAATCGGATAAACGGCATGTTTCGATACGTCTTGTTCGCCATGTTAATTTACTACAAATACAGTGTCCATGG
```

FIGURE 2 CONT'D

```
TF37    - MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPNQTAKAEKATTSYQDE -50
          :::::::::::::::::::::::::::::::::::: :     :   : :
TF205   - MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPNKTTGVSKEE--YKDV -48
TF37    - ETKKKTKEELDKLMEPALGYETQILRRNKAPKTETGEKRNERVVELSEDK -100
          :: :: :: :  ::::::::
TF205   - ETAKKEKEQLGELMEPALGYVVKVP------------------------- -73
TF37    - ITKLYQESVEIIPHLDELNGKTTSNDVYHSHDSKRLD------------- -137
                                              :  :  :
TF205   - -------------------------VSSFENKKVDISDIEVITNGNLD -96
TF37    - -----------------KNRDLKYVRSGYVYDG---SFNEIRRNDSGFH -166
                            :  : ::::::: ::    ::
TF205   - DVPYKANSSKYNYPDIKTKDSSLQYVRSGYVIDGEHSGSNE--------- -137
TF37    - VFKQGIDGYVYYLGVTPSKELPKGKVISYKGTWDFVSNINLEREIDGFDT -216
                ::::: :  : ::::        : : ::: :: ::
TF205   - ------KGYVYYKGNSPAKELPVNQLLTYTGSWDFTSNANL--------- -172
TF37    - SGDGKNVSATSITETVNRDHKVGEKLGDNEVKGVAH-------------- -252
                                         :  :
TF205   - ---------------------------NNEEGRPNYLNDDYYTKFIGKR -194
TF37    - ---------------SSEFAVDFDNKKLTGSLYRNGYINRNKAQEVTKRY -287
                          : : ::: :: :: :                   :
TF205   - VGLVSGDAKPAKHKYTSQFEVDFATKKMTGKL----------SDKEKTIY -234
TF37    - SIEADIAGNRFRGKAKA-------EKAGDPIFTDSNYLEGGFYGPKAEEM -330
             ::: :::: : : :                 ::  :::::::::::::
TF205   - TVNADIRGNRFTGAATASDKNKGKGESYNFFSADSQSLEGGFYGPKAEEM -284
TF37    - AGKFFTNNKSLFAVFAAKSENGETTTERIIDATKIDLTQFNAKELNNFGD -380
          ::::  : ::::::: ::         : ::::: ::::: :   :::::::
TF205   - AGKFVANDKSLFAVFSAKHNGSNVNTVRIIDASKIDLTNFSISELNNFGD -334
TF37    - ASVLIIDGQKIDLAGVNFKNSKTVEINGKTMVAVACCSNLEYMKFGQLWQ -430
          :::::::: :: :::  : :  ::::::::::::::::::::::::::::
TF205   - ASVLIIDGKKIKLAGSGFTNKHTIEINGKTMVAVACCSNLEYMKFGQLWQ -384
TF37    - KEGKQQVKDNSLFLQGERTATDKMPAGGNYKYVGTWDALVSKGTNWIAEA -480
                    :::::::::::::::: :::::: ::::: :::  :: : :
TF205   - QAEGGKPENNSLFLQGERTATDKMPKGGNYKYIGTWDAQVSKENNWVATA -434
TF37    - DNNRESGYRTEFDVNFSDKKVNGKLFDKGGVNPVFTVDATINGNGFIGSA -530
          :  :  :::::::: :  :   :::::: :::::::::: : :::: : :
TF205   - DDDRKAGYRTEFDVDFGNKNLSGKLFDKNGVNPVFTVDAKIDGNGFTGKA -484
TF37    - KTSDSGFALDAGSSQHGNAVFSDIKVNGGFYGPTAGELGGQFHHKSDNGS -580
          :::: ::::: :::    :  : :  : :::::::: :::::::::: :::
TF205   - KTSDEGFALDSGSSRYENVKFNDVAVSGGFYGPTAAELGGQFHHKSENGS -534
TF37    - VGAVFGAKRQIEK -593
          :::::::: :  :
TF205   - VGAVFGAKQQVKK -547
```

FIGURE 3

CTGTTATAGA TCTAGGAAAA GCAAGTTTAG GTTTGGACAT TATCTCTGGT
BglII
TTACTTTCTG GAGCATCTGC AGGTCTCATT TTAGCAGATA AAGAGGCTTC

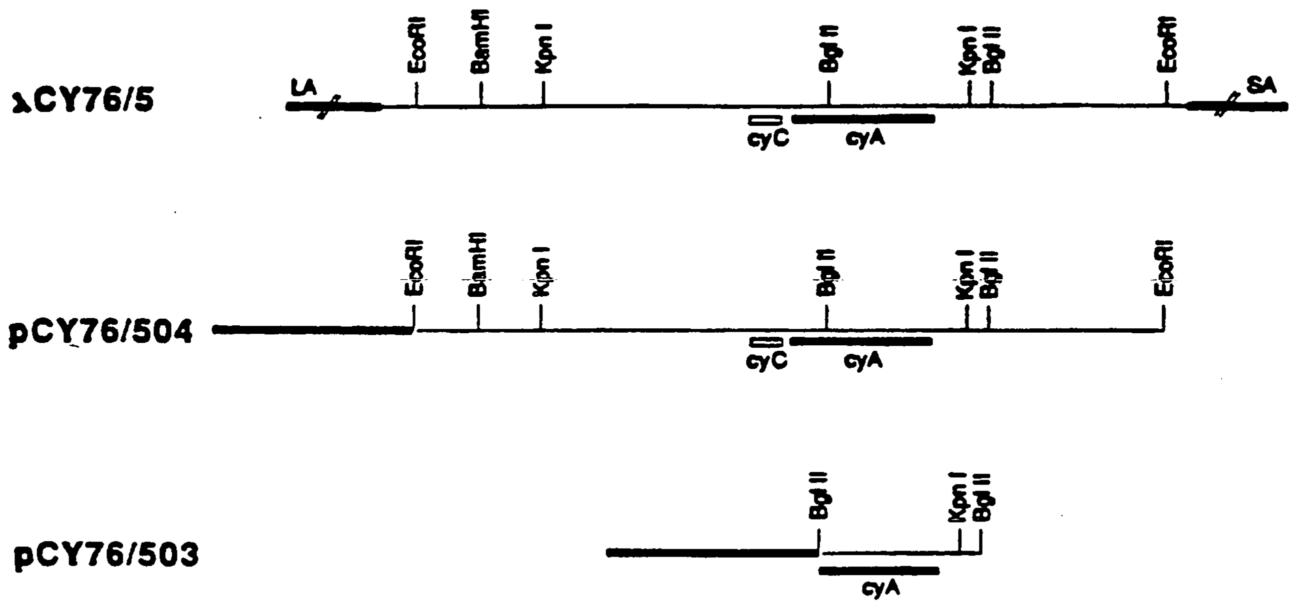
AACAGAAAAG AAAGCTGCCG CAGGTGTAGA ATTTGCTAAC CAAATTATAG

GTAATGTAAC AAAAGCGGTC TCATCTTACA TTCTTGCCCA ACGAGTCGCT

TCAGGTTTGT CTTCAACTGG TCCTGTCGCT GCATTAATCG CATCTACAGT

TGCACTAGCT GTTAG

FIGURE 4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LA
SA
EcoRI
BamHI
Kpn I
Bgl II
Kpn I
Bgl II
EcoRI
cyC
cyA
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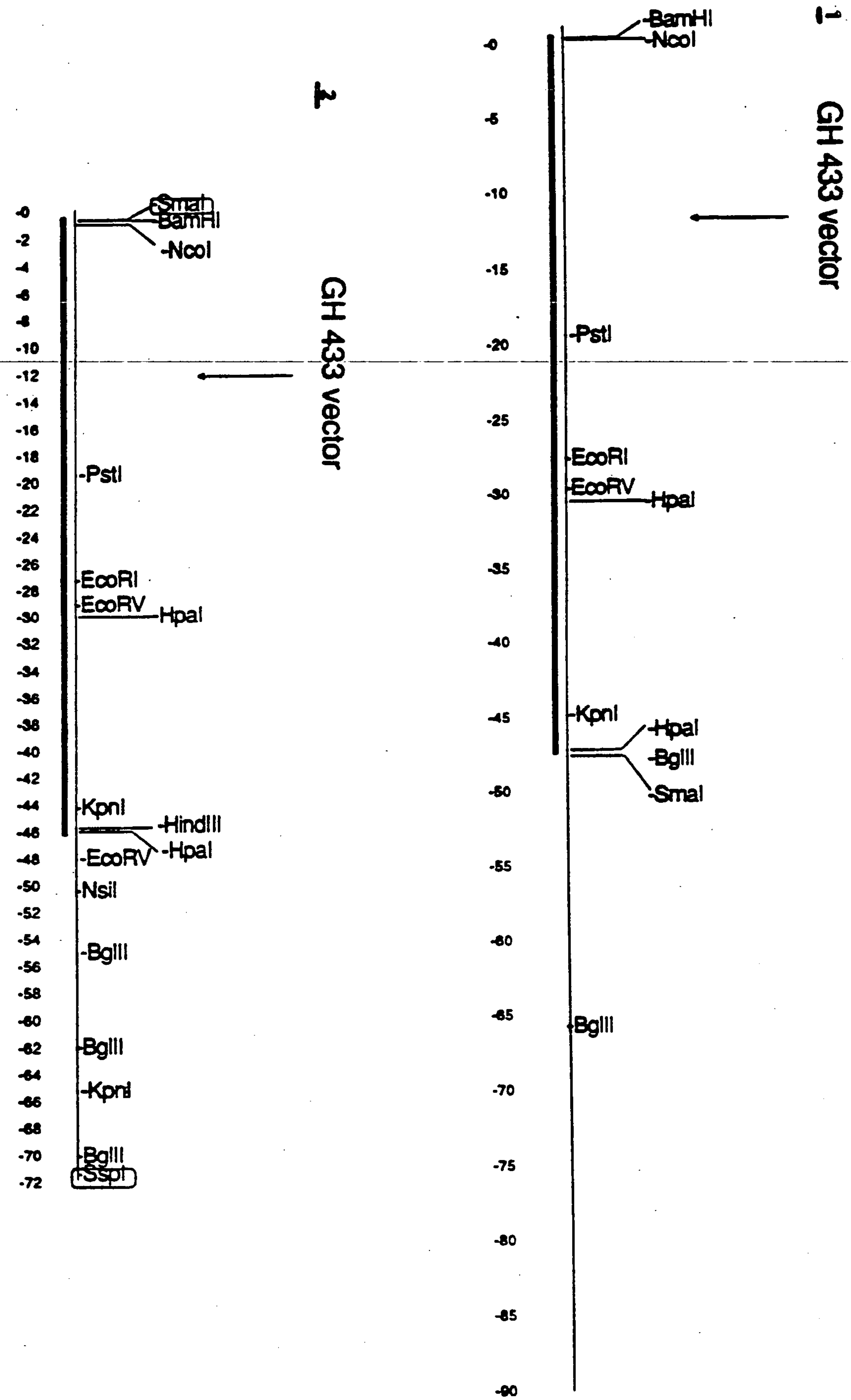
EcoRI
BamHI
Kpn I
Bgl II
Kpn I
Bgl II
EcoRI
cyC
cyA
pCY76/503
Bgl II
Kpn I
Bgl II
cyA

FIGURE 5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GH 433 vector
-BamHI
-NcoI
-PstI
-EcoRI
-EcoRV
-HpaI
-KpnI
-HpaI
-BglII
-SmaI
-BglII
2
GH 433 vector
-SmaI
-BamHI
-NcoI
-PstI
-EcoRI
-EcoRV
-HpaI
-KpnI
-HindIII
-EcoRV
-HpaI
-NsiI
-BglII
-BglII
-KpnI
-BglII
-SspI

FIGURE 6

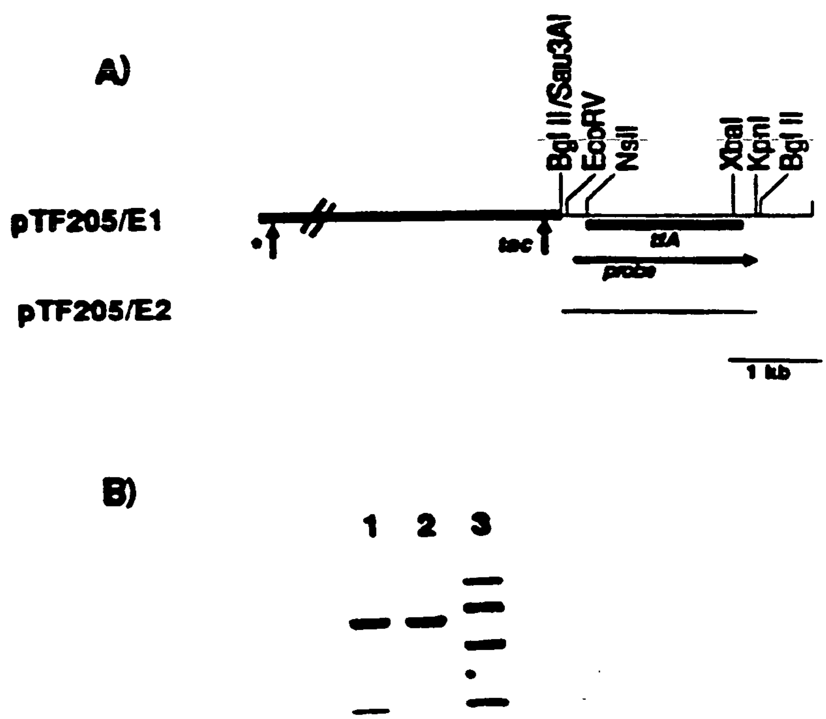

A)
Bgl II/Sau3AI
EcoRV
NsiI
XbaI
KpnI
Bgl II
pTF205/E1
tac
probe
pTF205/E2
1 kb
B)
1 2 3

FIGURE 7

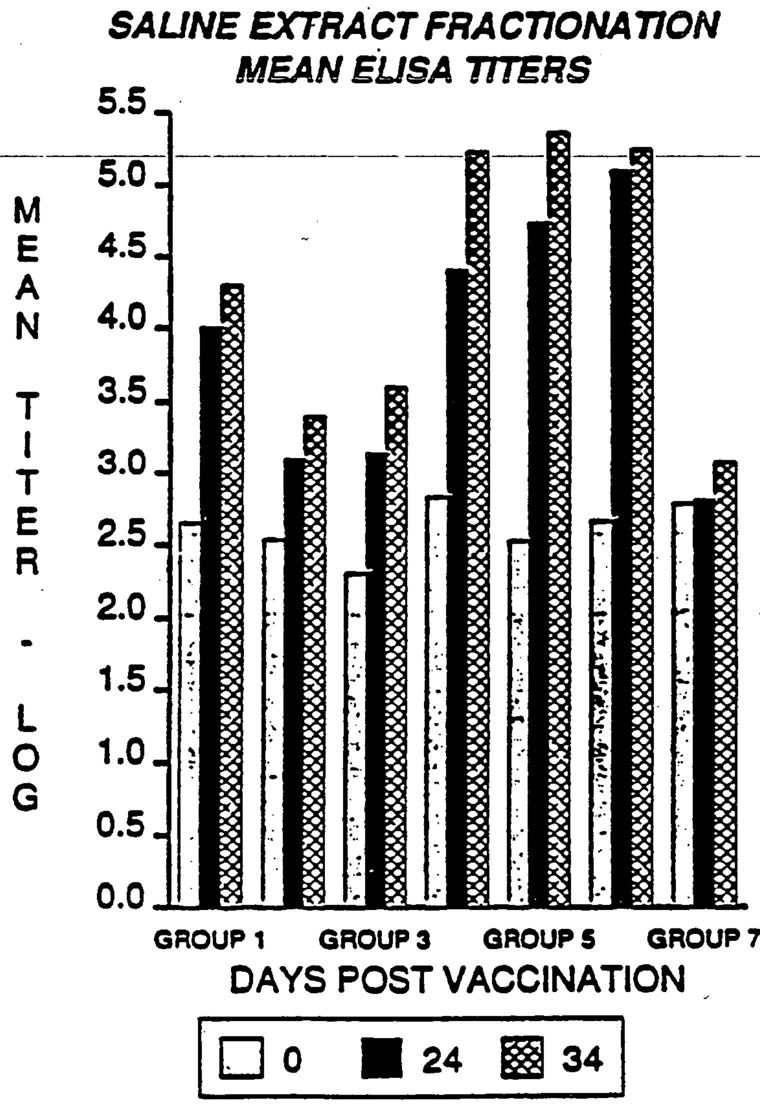

SALINE EXTRACT FRACTIONATION
MEAN ELISA TITERS
MEAN TITER - LOG
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GROUP 1
GROUP 3
GROUP 5
GROUP 7
DAYS POST VACCINATION
0
24
34

FIGURE 8

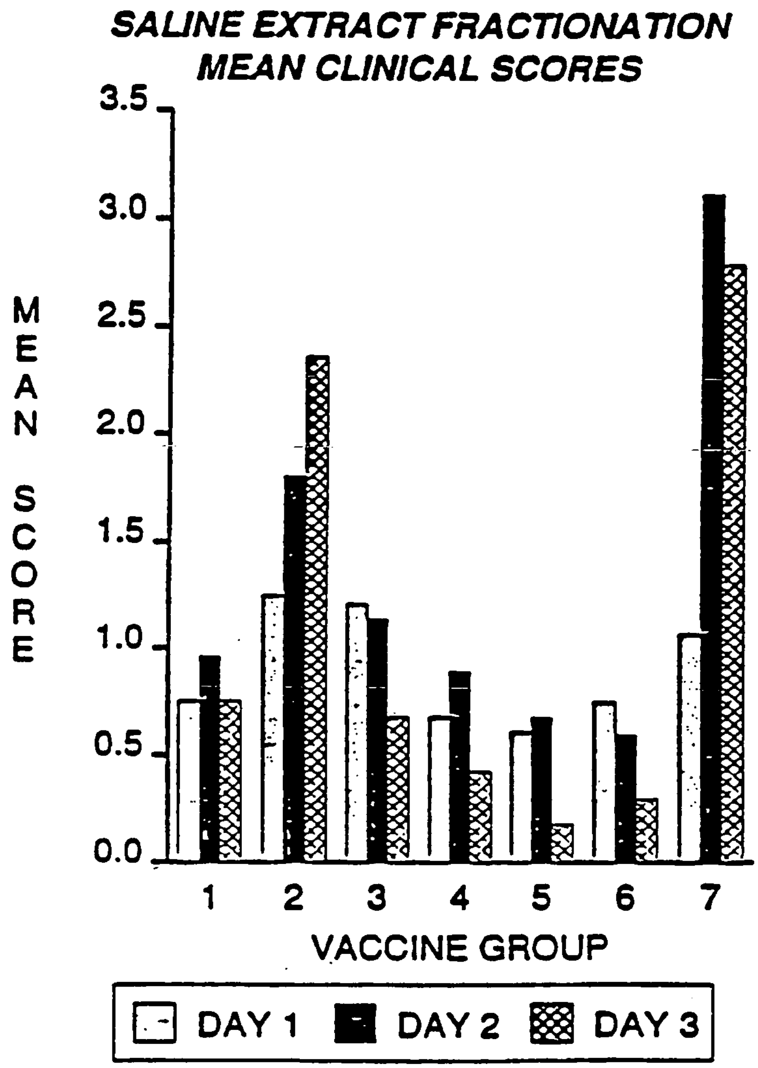

SALINE EXTRACT FRACTIONATION
MEAN CLINICAL SCORES
MEAN SCORE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
VACCINE GROUP
DAY 1
DAY 2
DAY 3

FIGURE 9

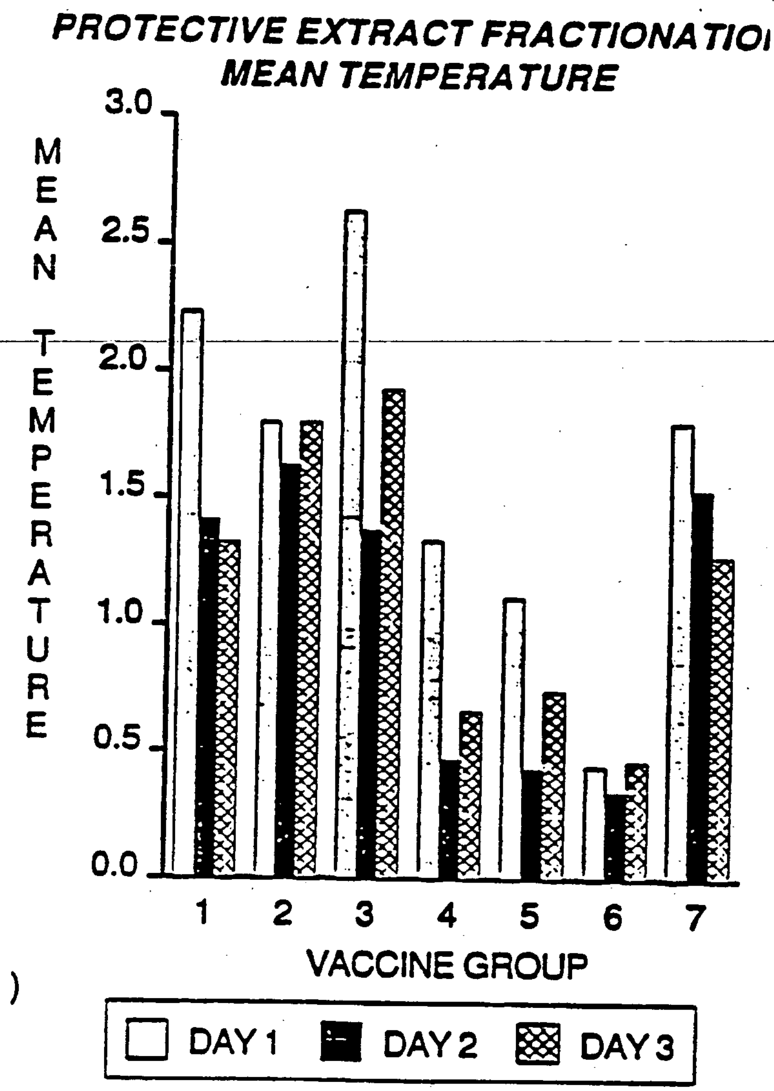

PROTECTIVE EXTRACT FRACTIONATIOI
MEAN TEMPERATURE
MEAN TEMPERATURE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
VACCINE GROUP
DAY 1
DAY 2
DAY 3

FIGURE 10

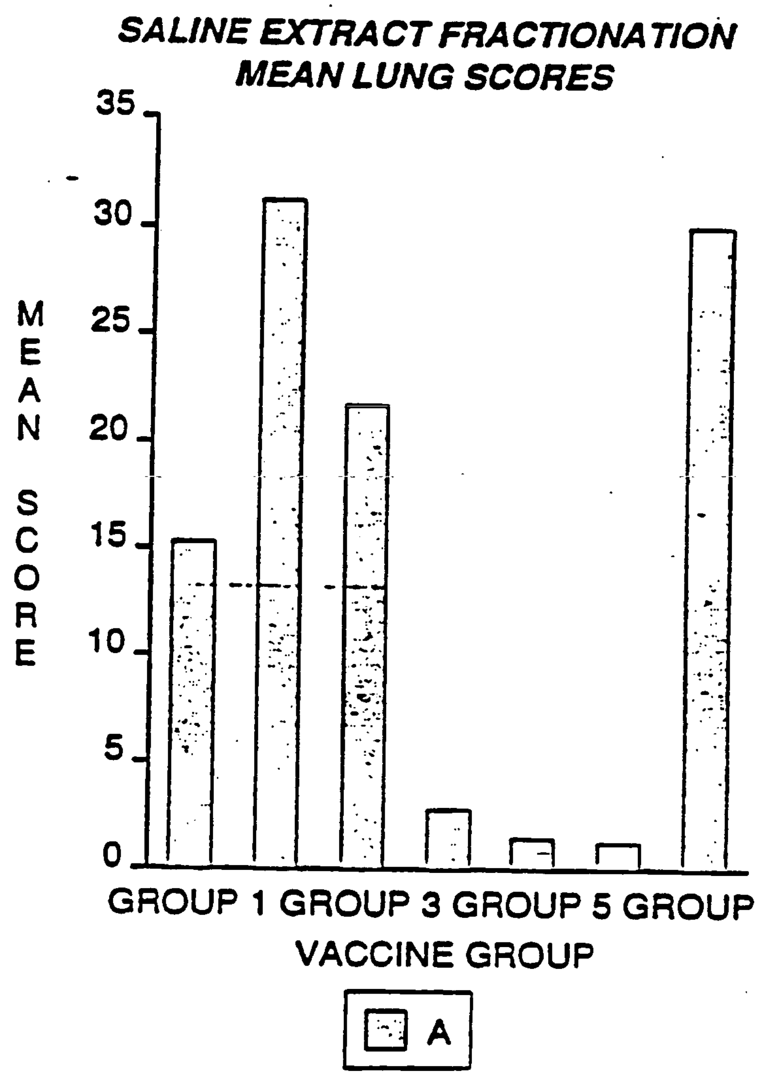

SALINE EXTRACT FRACTIONATION
MEAN LUNG SCORES
MEAN SCORE
35
30
25
20
15
10
5
0
GROUP 1 GROUP 3 GROUP 5 GROUP
VACCINE GROUP
A

FIGURE 11

Clinical Response After Challenge

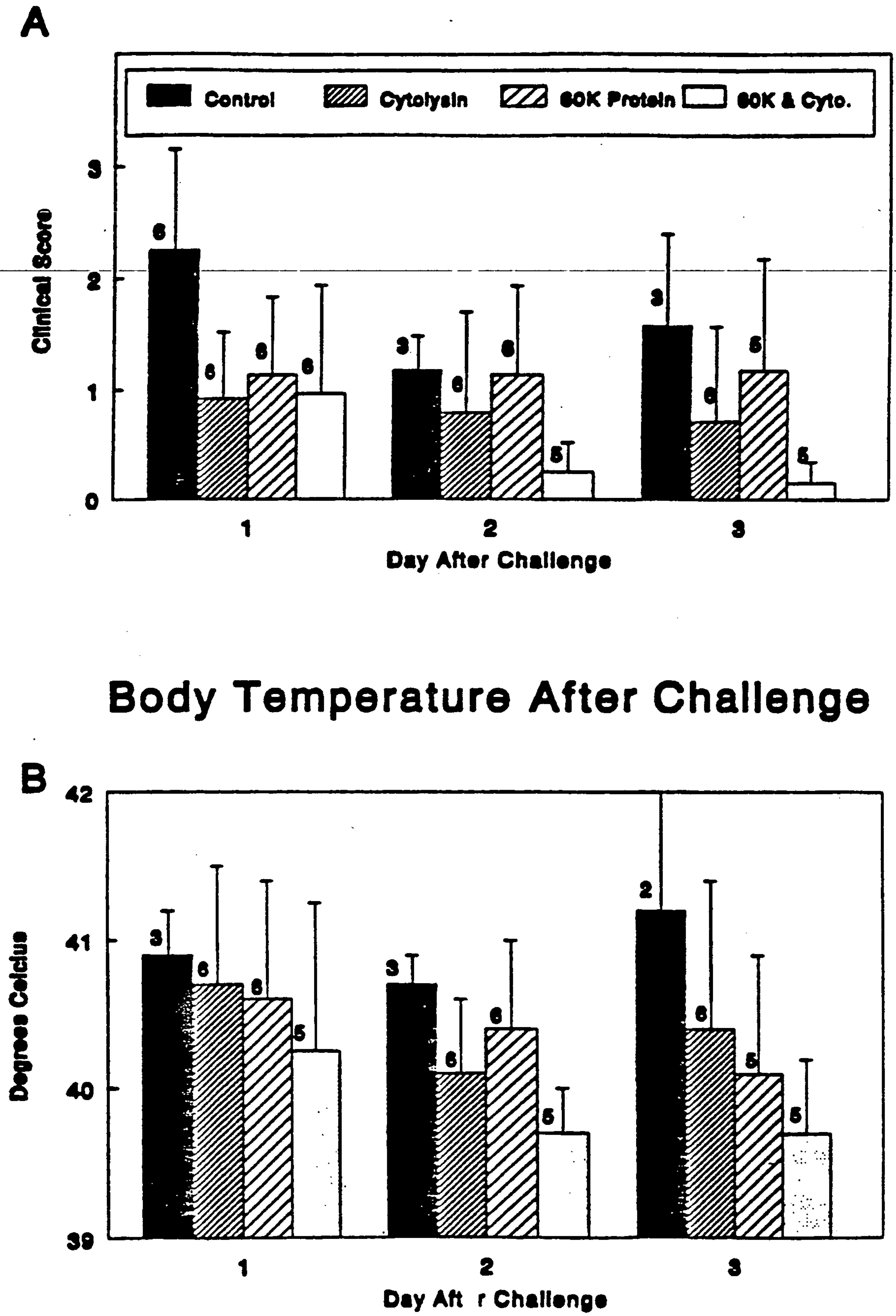
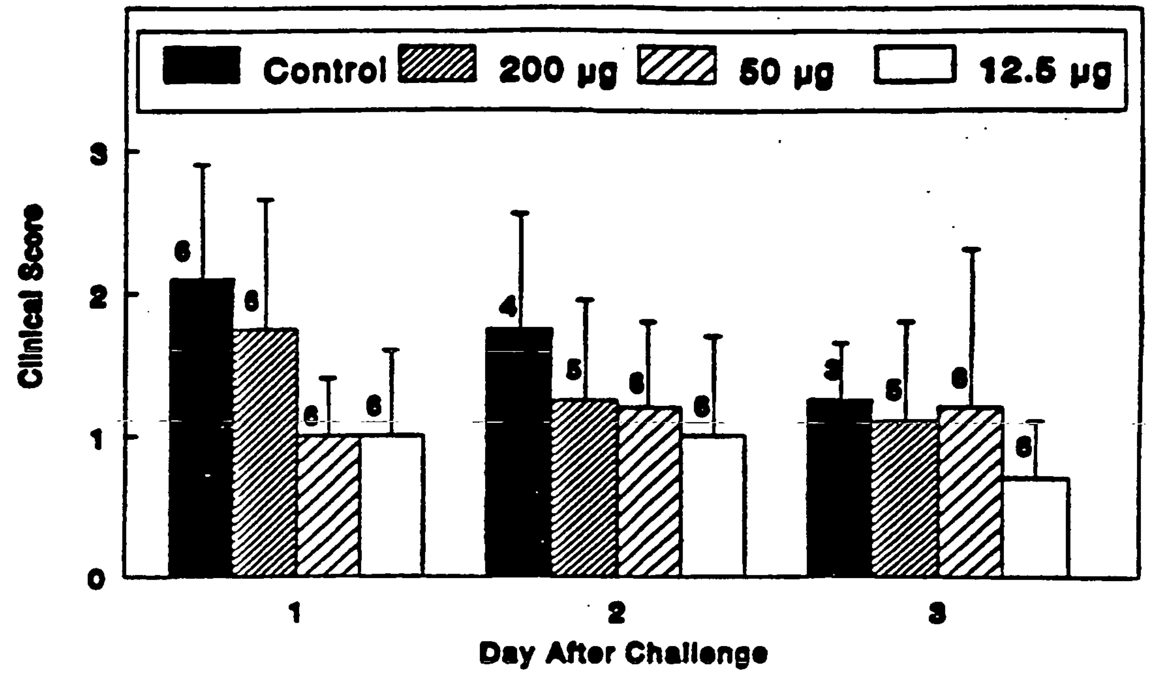


FIGURE 12

Clinical Response After Challenge

A



Body Temperature After Challenge

B

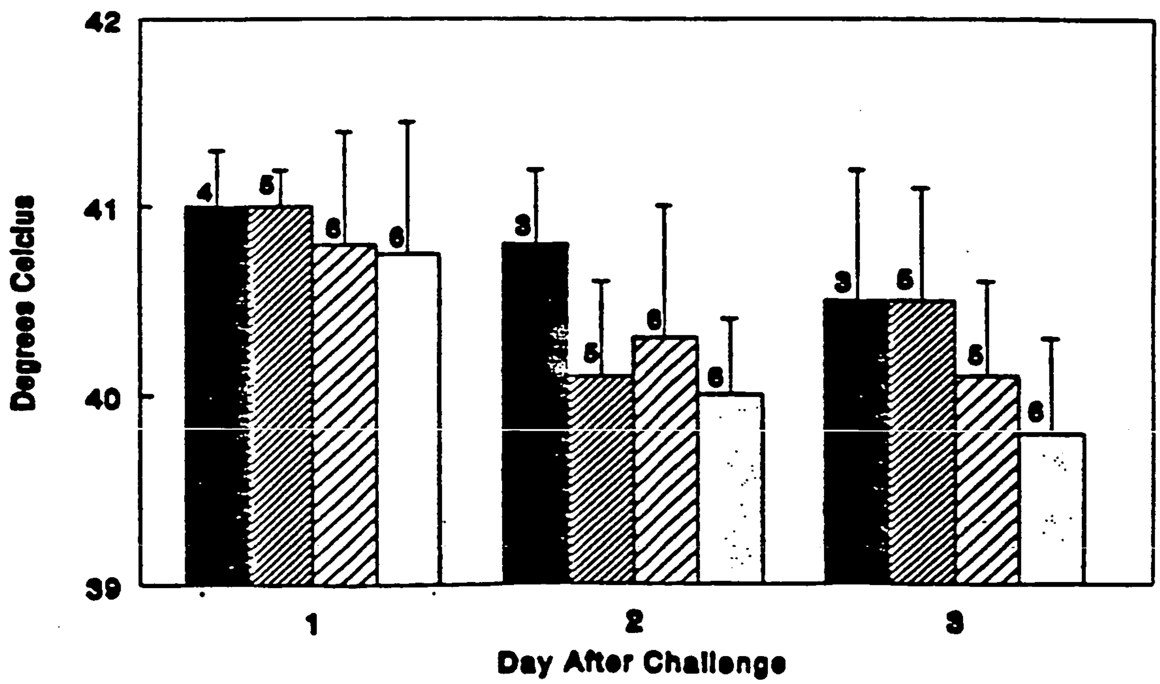


FIGURE 13

a

```
BamHI ----//----|<----------|CTTAATGATA TAACAGCGGT CAAATTCTAA
                  1201 bp repeat
AATCTTTTGC AATGTGCAAC TTTTATTAGG ATT -----//------ cytA -//-
TCTAGATGGA AAAGGTTTGT CTTTAACATC ATGGTTAATC GCAGCAAAAT CATTAGATTT
 XbaI
AAAAGCAAAG GCTATTAATA AAGCCGTTGA GCGTTTACCT TTTGTTAATT TACCTGCACT
TATCTGGAGG GAAGATGGAA AACATTTTAT CTTAGTAAAG ATAGATAAAG ATAAAAAACG
CTATTTAAC|<----------|---//---BglII
           1201 bp repeat
```

FIGURE 14

b

```
diverging sequence
|
T-G-T-A-G-A-A-A-A-T-C-A-A-A-C-C-T-A-A-T-C-T-G-A-C-A-┐
                                                    repeat sequence
A-C-A-T-C-T-T-C-T-A-G-T-C-T-G-A-A-C-T-A-G-A-C-T-G-T-┘
|
diverging sequence
```

FIGURE 15

```
   1 GGATCCTGTT CTTGGTGAAA GTGTGGAACT TAAAGTTAAC TTATGTTTAG AGAAAAAAGG
     BamHI
  61 ATGGTATCTA GAGCAAGGTC CAGTGTGTGA AGAAAAATAC GTATGGAATG AACCGGAATG
 121 TATTAAATGG CGAGCAAAAT ATAGTAAGCC AAATGTGCAA CCTTGGGGAT AATAGTCATT
 181 TAAGTGTTTT AAAAATTTAA TTTCAGAAAT TTGTAATGGA TACAATGAAT ACAGAAAATA
 241 ATTAATGTTT AAAATCAAGC ACTAAATGAT TTTGTAATGG CACTTTAGCT GGGGTTATAT
 301 GAAGTAAATT CTTAATGTGT AGAAAATCAA ACCTAATCTG ACAGTTCCCG TTTAAAATTA
                            inverted repeat
 361 CCGTGTCTGT CAGATTAATT TGAGCTTAAA TTCTTTTCTG CCCAAATCCG TTTTCCATCA
                     *** <------ end of open reading frame
 421 AGTAATGTTG CCATCGGTGT TCTGCCACAG CACACTTTTC CTTGATGTGT TCGATGGTGA
 481 TTATAATACA TTAACCACTC ATCTAAATCA GCTTGTAATG TCGCTAAATC CGTATATATT
 541 TTCTTCCTAA ATGCGACTTG GTAAAATTCT TGTAAGATAG TCTTATGAAA ACGTTCACAG
 601 ATACCATTCG TCTGTGGATG CTTCACTTTC GTTTTAGTAT GCTCTATGTC ATTTATCGCT
 661 AAATAAAGCT CATAATCGTG ATTTTCCACT TTGCCACAAT ATTCACTGCC ACGGTCGGTG
 721 AGAATACGCA ACATCGGTAA TCCTTGGGCT TCAAAGAACG GCAGTACTTT ATCATTGAGC
 781 ATATCTGCAG CGGCAATTGC GGTTTTCATT GTGTAGAGCT TTGCAAAAGC AACCTTACTA
 841 TAAGTATCAA CAAATGTTTG CTGATAAATG CGTCCAACAC CTTTTAAATT ACCTACATAA
 901 AAGGTATCTT GTGAACCTAA ATAGCCCGGA TGAGCGGTTT CAATTTCTCC ACTCGATATA
 961 TCATCCTCTT TCTTACGTTC TAGGGCTTGG ACTTGACTTT CATTTAGAAT AATGCCTTTC
1021 TCAGCCACTT CTTTCTCTAG TGCATTTAAA CGCTGTTTAA AGTTAGTAAG ATTATGACGT
1081 AGCCAAATGG AACGAACACC ACCGGCTGAA ACAAACACAC CTTGCTTGCG AAGTTCGTTA
1141 CTCACTCGAA CTTGTCCGTA AGCTGGAAAA TCTAGAGCAA ATTTTACAAC AGCTTGCTCA
1201 ATGTGCTCGT CTACTCGATT TTTGATATTC GGTACCCGAC GAGTTTGCTT AACTAATGCT
                                  KpnI
1261 TCAACACCGC CTTGCGCTAC GGCTTGTTGA TAGCGATAGA ATGTATCTCG GCTCATTCCC
1321 ATCGCTTTAC AAGCTTGAGA AATGTTTCCG AGTTCTTCTG CTAAATTGAG TAAACCGGTC
1381 TTGTGTTTAA TGAGCGGATT GTTAGAATAA AACATGAGAG TTTCCTTTTT TGTTTAGATT
       start of open reading frame <--- MET         SD
1441 GAATTTTAGA CACTCATATT CTAAACGGGA AACTCTCATT TTTATAATGA TTTGTCAGAT
1501 CAAGTCTGAT CTTCTACAAA TATTATCCCC ATTTATGGAG TTCGTCTTTT AGATGAACTC
     inverted repeat
1561 CTATTGTTTA TAATTCGATA AAATTAGCTT TCTCACAGCA ACTCAGCAAT GGGTTGCTTT
1621 TTTATTTGAC AGAAAAACAA CGTAGATCT
                            BglII
```

FIGURE 16

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 92/00460

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; A61K39/102; C12N1/21; C07K13/00
//(C12N1/21,C12R1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 106 653 (THE TEXAS A&M UNIVERSITY SYSTEM) 16 May 1991	1,8-9, 15, 27-28, 32-34
Y	see figure 1 ---	12
X	DNA vol. 8, no. 9, November 1989, NEW YORK, USA pages 635 - 647 CHANG, Y.-F. ET AL. 'Cloning and characterization of a hemolysin gene from Actinobacillus (Haemophilus) pleuropneumoniae' cited in the application see the whole document --- -/--	27-28, 32-33

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

2

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 JANUARY 1993	22.01.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y A	WO,A,9 012 591 (UNIVERSITY TECHNOLOGIES INTERNATIONAL INC.) 1 November 1990 see the whole document ---	1-2,15, 34 12 1-7, 35-37
X	EP,A,0 420 743 (RHÔNE MERIEUX) 3 April 1991 see the whole document ---	1,8,15
X	INFECTION AND IMMUNITY vol. 59, no. 9, September 1991, WASHINGTON US pages 3026 - 3032 FREY, J. ET AL. 'Nucleotide sequence of the hemolysin I gene from Actinobacillus pleuropneumoniae' cited in the application see the whole document ---	27,32-33
P,X	INFECTION AND IMMUNITY vol. 60, no. 8, August 1992, WASHINGTON US pages 3253 - 3261 GERLACH, G.-F. ET AL. 'Characterization of two genes encoding distinct transferrin-binding proteins in different Actinobacillus pleuropneumoniae isolates' see the whole document ---	16-18, 23-26, 32-33, 35,37
P,X	INFECTION AND IMMUNITY vol. 60, no. 3, March 1992, WASHINGTON US pages 892 - 898 GERLACH, G.-F. ET AL. 'Cloning and expression of a transferrin-binding protein from Actinobacillus pleuropneumoniae' see the whole document ---	16-17, 23-24, 32-33,35
P,X	VACCINE vol. 10, no. 8, 1992, GUILDFORD GB pages 512 - 518 ROSSI-CAMPOS, A. ET AL. 'Immunization of pigs against Actinobacillus pleuropneumoniae with two recombinant protein preparations' see the whole document --- -/--	1-4,8-9, 13, 15-17, 23-24, 27-28, 32-34

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	INFECTION AND IMMUNITY vol. 59, no. 11, November 1991, WASHINGTON US pages 4110 - 4116 ANDERSON, C. ET AL. 'Isolation and molecular characterization of spontaneously occuring cytolysin-negative mutants of Actinobacillus pleuropneumoniae serotype 7' see the whole document -----	27-28, 32-33

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 34 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9200460 SA 65461

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 07/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9106653	16-05-91	AU-A-	6751390	31-05-91
		EP-A-	0500736	02-09-92
WO-A-9012591	01-11-90	AU-A-	5526190	16-11-90
		US-A-	5141743	25-08-92
EP-A-0420743	03-04-91	FR-A-	2652266	29-03-91
		AU-A-	6523090	28-04-91
		CA-A-	2035474	27-03-91
		WO-A-	9104747	18-04-91
		JP-T-	4502018	09-04-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82